The *SEC1* and *SEC5* genes of *Saccharomyces cerevisiae*.

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I dedicate this thesis to Mr and Mrs N. R. Egerton, "Mum and Dad",
for their endless support and encouragement during the last 24 years;
and to my wife Jacquelyn, whose understanding and belief
in me has made this thesis possible.

Declaration.

This study was carried out under the supervision of Dr. Alan Boyd at the Leicester Biocentre, University of Leicester between October 1985 and June 1987; and then in the Department of Biochemistry, University of Edinburgh between July 1987 and September 1988.

The experimental work presented in this thesis, unless stated otherwise, is my own; and this manuscript presented here has been composed by myself.

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Abstract.

Proteins that are secreted from the yeast, *Saccharomyces cerevisiae*, traverse a pathway similiar to that found in mammalian cells: nascent polypeptides enter the secretory pathway by translocation across the endoplasmic reticulum (ER) membrane, after which they are sequentially transported through the Golgi complex, where they are packaged into secretory vesicles which ultimately fuse with the plasma membrane.

Many of the cellular functions which contribute to the secretion pathway have been genetically defined, by the isolation of temperature-sensitive lethal mutants (*sec*) which are deficient in protein secretion. At the restrictive temperature (37°C) *sec* mutants exhibit an accumulation of secretory glycoproteins inside an organelle of the secretory apparatus, ie either ER, Golgi, or secretory vesicles; but on return to a permissive temperature accumulated material is secreted. Many of the *sec* mutants are also incapable of fluid phase endocytosis at 37°C, indicating a general requirement for *SEC* gene products during intracellular movement of membrane.

The *sec1-1* mutation results in an accumulation of secretory vesicles and cessation of endocytosis at 37°C. This thesis describes the isolation of a fragment of yeast genomic DNA that complements the *sec1-1* mutation. Transposon mutagenesis and subcloning experiments have localised the gene responsible for complementation within this fragment, and the nucleotide sequence spanning this region has been determined. A single open reading frame of 1878 bp has been identified, which could encode a polypeptide of 626 amino acids.

Previous genetic analyses mapped the *SEC1* gene in a tightly linked cluster of genes on chromosome IV, including *SEC5, SEC7,* and *CDC37.* Intriguingly, mutations in the *SEC5* gene, which is immediately adjacent to *SEC1*, result in a similiar phenotype to that exhibited by a *sec1-1* mutant. In this thesis I demonstrate that the yeast genomic DNA fragment isolated from the genomic library also harbours a second gene, independent of the *SEC1* gene, which can complement a *sec5-24* mutation. Further genetic analysis has revealed a relationship between the two genes: at a restrictive temperature of 33.5°C a *sec5-24* mutant can be rescued by the introduction of multiple copies of the *SEC1* gene; this interaction is unidirectional however, since a *sec1-1* mutant cannot be rescued by multiple copies of the *SEC5* gene. This phenotype is not due to sequence similarites in the two gene products, since the nucleotide sequences of the *SEC1* and *SEC5* genes do not bear any resemblance.

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Abbreviations.

ACTH	adrenocorticotrophic hormone
ASGP	asialoglycoprotein
ATP	adenosine 5'-triphosphate
bp	base pair
BSA	bovine serum albumin
Ci	Curie
cpm	counts per minute
CPY	carboxypeptidase Y
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
EDTA	diaminoethanetetra-acetic acid
EGTA	1,2-di(2-aminoethoxy)ethane-N,N,N',N'-tetra acetic acid
ER	endoplasmic reticulum
GTP	guanosine 5'-triphosphate
hr	hour
IPTG	isopropylthiogalactoside
kb	kilobase
LDL	low density lipoprotein
min	minute
MOPS	morpholinopropanesulphonic acid
NEM	N-ethylmaleimide
NSF	N-ethylmaleimide sensitive factor
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
POMC	proopromelancortin
PPO	2,5 diphenyloxazole
PVP	polyvinylpyrrolidone
RER	rough endoplasmic reticulum
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
sec	second
SRP	signal recognition particle
SV	secretory vesicle
TGN	*trans* Golgi network
Tris	2-amino-2-(hydroxymethyl) propane-1,3-diol(tris)
X-GAL	5-bromo-4-chloro-indolyl-ß-D-galactoside

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Introduction.

1. INTRODUCTION

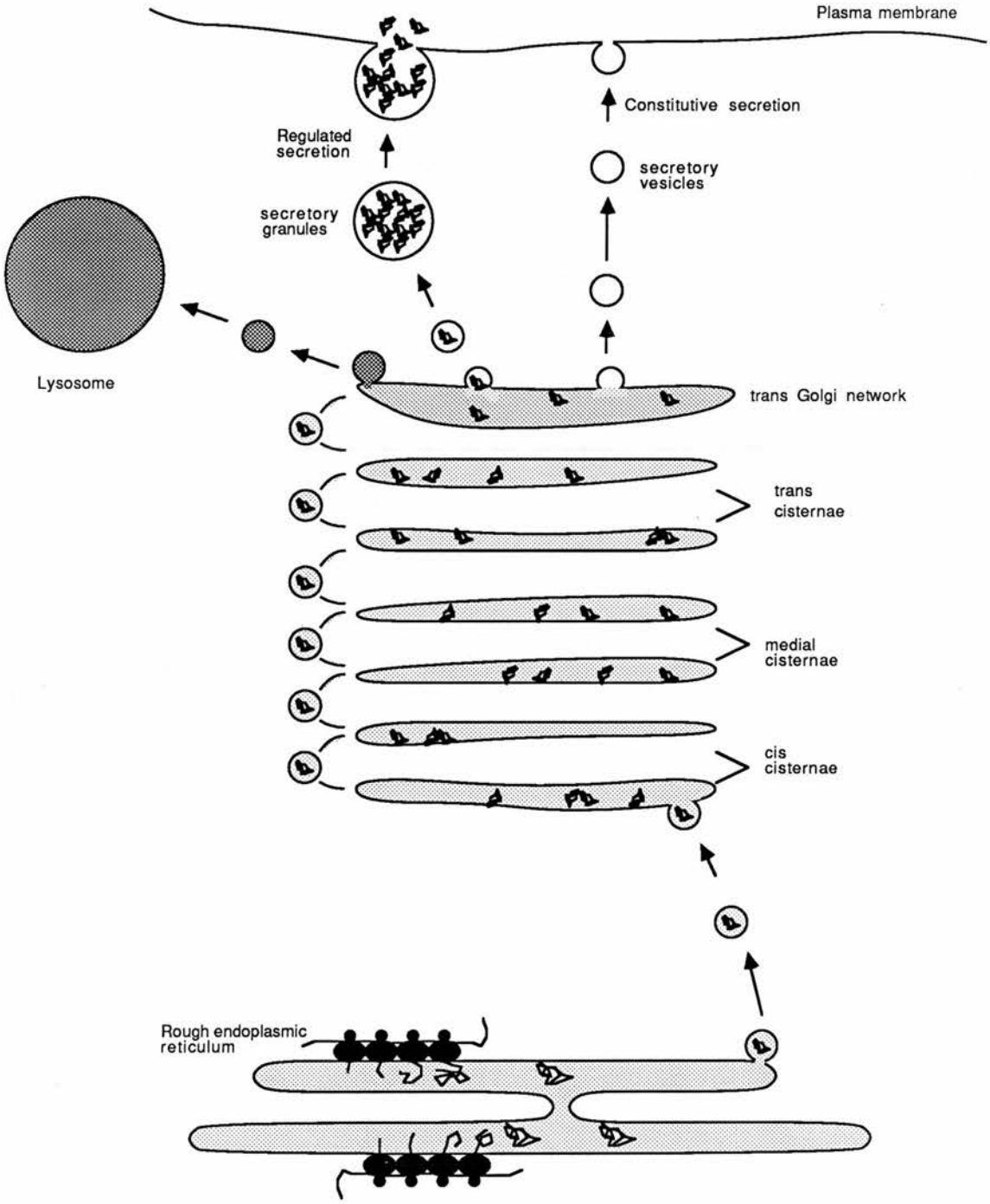
In this thesis I describe the characterisation of two genes from *Saccharomyces cerevisiae* whose products are thought to participate in the late stages of secretion. This introduction is therefore designed to provide the reader with a sound understanding of protein secretion from yeast. The current model of protein secretion, however, is largely derived from biochemical investigation of mammalian systems; I have therefore reviewed this model since it provides a conceptual framework in which investigations of the yeast secretory pathway can be interpreted. Throughout this review I have chosen to highlight certain areas of investigation that may be directly relevant to this project. Also, investigation of the mammalian endocytic pathway has provided many insights into the mechanisms employed during intracellular membrane transport. Recent biochemical and genetic evidence indicates that the secretory pathway could share many of these characteristics, I have therefore reviewed the endocytic pathway, again choosing some aspects that I think may be relevant to this thesis.

1.1 SECRETION OF PROTEINS FROM MAMMALIAN CELLS: A BRIEF OVERVIEW.

For eukaryotes, the generally accepted view is that soluble and membrane proteins move from the endoplasmic reticulum (ER) to the Golgi complex, where they are packaged into secretory vesicles and transported to the plasma membrane (See fig. 1.1, Palade 1975). Four classes of proteins traverse the secretory pathway; these are proteins to be secreted, lysosomal proteins, plasma membrane proteins, and proteins of the secretory apparatus itself - both soluble and membrane proteins. All these different types of protein are synthesised on polysomes attached to the rough ER (Siekevitz and Palade 1960). Simultaneous to their synthesis, these proteins are translocated across the ER membrane, which ultimately allows the cell to segregate metabolic pathways, communicate with other cells, and build new membrane.

Figure 1.1 **The mammalian secretory pathway.**

This figure is a diagrammatic representation of a generalised mammalian secretory pathway. Proteins enter the pathway by co-translational translocation across the ER membrane, and are then transported to the Golgi complex, which they enter at the *cis* face, and sequentially traverse the stacked cisternae until they reach the TGN where they are packaged into vesicles for delivery to their destination. In this diagram the Golgi complex is depicted as having two *cis*, two medial, and two *trans* cisternae, this number is arbitrary and does vary. Transport between all the different compartments of the secretory pathway is proposed to oocur by vesicular translocation. During this journey proteins may be modified by addition of oligosaccharide, formation of disulphide bonds, phosphorylation, sulphation, or proteolytic cleavage (see text for further details). Proteins that are destined for alternative locations in the cell, ie. soluble and membrane proteins of secretory organelles and the lysosome, and proteins that are released by the regulated secretory pathway are thought to be segregated from proteins that are constitutively released during passage through the Golgi. See text for further details.


Plasma membrane
Constitutive secretion
Regulated secretion
secretory vesicles
secretory granules
Lysosome
trans Golgi network
trans cisternae
medial cisternae
cis cisternae
Rough endoplasmic reticulum

1.1.1 Translocation of proteins across the ER membrane.

Proteins that are destined to be translocated into the secretory pathway possess a specific signal sequence, which is usually located at the extreme amino-terminus of the protein (Blobel and Dobberstein 1975). No consensus for a signal sequence exists although the following features are commonly found: 1-3 positively charged amino acids in the amino-terminal region of the signal peptide; followed by a long hydrophobic sequence consisting of 14-20 amino acids; and finally a glycine or alanine residue at the carboxy-terminus of the signal peptide (for review see Duffaud *et al.,* 1985). As the signal sequence emerges from the large subunit of the ribosome it is recognised and bound by a cytoplasmic ribonucleoprotein complex (the signal recoginition particle, SRP), which arrests further elongation of the polypeptide chain. The ribosome-SRP-nascent chain complex is subsequently directed to the membrane of the ER via a direct interaction between SRP and a receptor protein (SRP receptor or docking protein). Following attachment to the membrane both SRP and docking protein are released from the complex, and elongation of the nascent polypeptide continues with the growing polypeptide being directed across the membrane (for more details of translocation, SRP, and the SRP receptor see review by Walter and Lingappa (1986) and references therein). Proteins will usually pass completely through the membrane into the lumen of the ER unless they possess a second hydrophobic sequence which can anchor the protein in the membrane (for review see Rapoport and Weidmann 1985).

1.1.2 Modifications of proteins within the ER.

During translocation the polypeptide is modified in a variet of ways by enzymes localised on the luminal side of the ER membrane. The signal peptide is usually removed as it emerges through the membrane, by the enzyme signal peptidase (Baker *et al.*, 1986, Evans *et al.*, 1986); oligosaccharide chains are added to selected asparagine residues (see below and for review see Kornfeld and Kornfeld 1985), and

oxidation of cysteine residues occurs to form disulphide bonds (Bergmann and Kuehl 1979, Peters and Davidson 1982).

Most secreted proteins become glycosylated by addition of sugar residues to the polypeptide chain. The biochemical reactions involved in the addition of oligosaccharides to asparagine residues have been elucidated, and are often used as evidence for the progression of a polypeptide through the secretory pathway (see fig. 1.2). N-linked glycosylation is initiated in the ER: a core oligosaccharide chain consisting of three glucose, nine mannose, and two N-acetylglucosamine molecules ($Glc_3Man_9GlcNAc_2$) is transferred from a lipid donor to the amide nitrogen of an asparagine residue (hence the name N-linked glycosylation). Prior to exit from the ER one mannose and the three glucose molecules are removed by the action of a mannosidase and glucosidases respectively. More extensive modification of these oligosaccharides takes place in the Golgi and is described below. The asparagine residue to be glycosylated almost always occurs in the sequence Asn-X-Thr/Ser, where X can be any amino acid except possibly proline and aspartic acid, but not all Asn-X-Ser/Thr sequences are glycosylated.

Once within the lumen of the ER, nascent polypeptide chains must fold or assemble into a competent structure prior to exit from the ER and transport to the Golgi; proteins which fail to do so are prevented from leaving. In the few cases studied such retained proteins are more often than not found in association with a resident ER protein of molecular weight 78K, termed BiP or glucose regulated protein 78 (GRP78, Munro and Pelham 1986), whose synthesis is actually increased by the presence in the ER of malfolded proteins (Kozutsumi *et al.*, 1988). GRP78 was first observed in myeloma cells in association with immunoglobulin heavy chains that were being synthesised in cells which lacked light chains (Haas and Wable 1983), but it has since been found in association with malfolded viral haemagglutinin (Gething *et al.*, 1986), factor VIII, von Willebrand factor, and tissue plasminogen activator (Dorner *et al.*, 1987). Whether GRP78 actually helps in the folding of

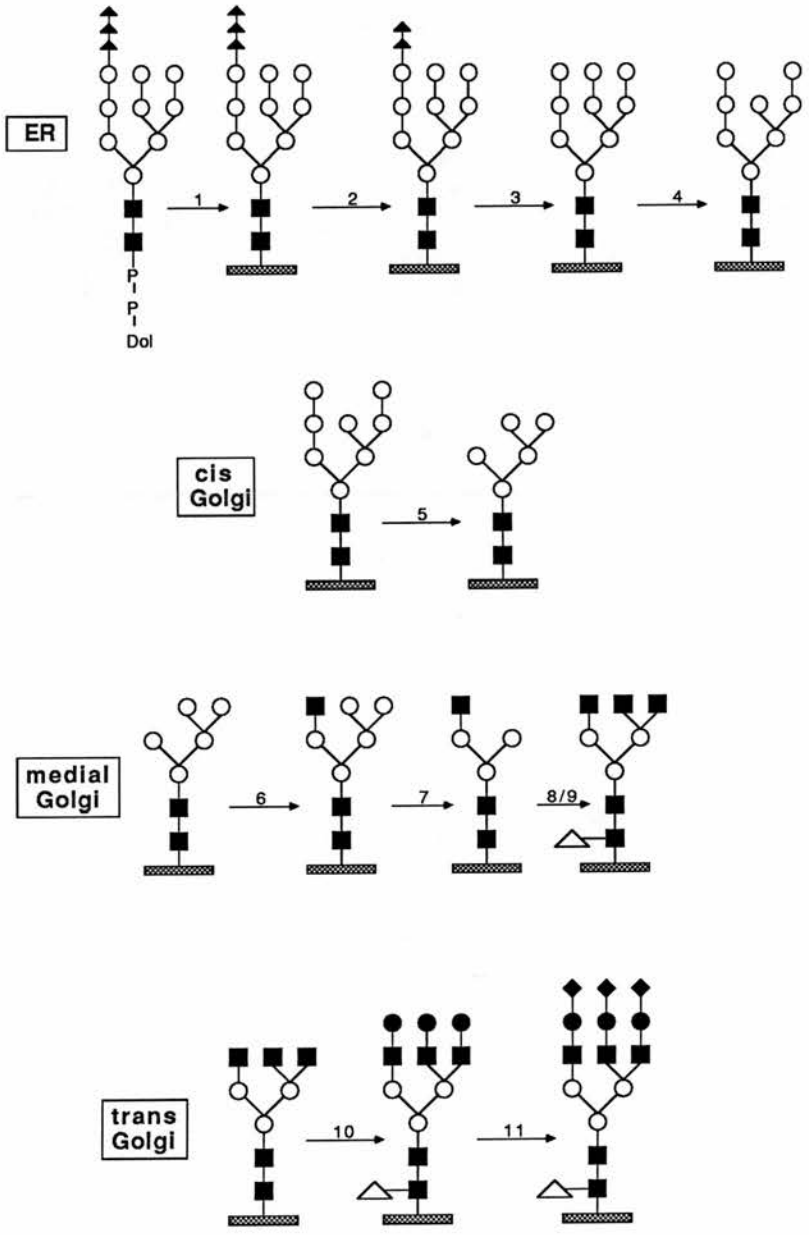
Figure 1.2 **N-linked glycosylation.**

This figure is a diagrammatic representation of N-linked glycosyaltion through the secretory pathway. Newly synthesised proteins may be modified by addition of oligosaccharides to the asparagine residue in the sequences Asn-X-Ser/Thr. During translocation of the polypeptide across the membrane of the ER an oligosaccharide moiety is transferred from dolicholpyrophosphate (Dol-P-P) by oligosaccharyltransferase (1). This 'core' oligosaccharide is processed prior to leaving the ER: three molecules of glucose and one of mannose are removed by the sequential action of α-glucosidase I (2), α-glucosidase II (3), and ER 1,2 mannosidase (4). The oligosaccharide chain undergoes further sequential modification during transit through the Golgi apparatus; each modification has been demonstrated to occur in a particular cisterna, which is a major piece of evidence for functional compartmentalisation of the Golgi:

cis cisternae: oligosaccharide side chains are trimmed by removal of four mannose residues by Golgi α-mannosidase I (5).

medial cisternae: sequentially, a molecule of N-acetylglucosamine is added by N-acetylglucosaminyltransferase I (6), followed by removal of one more mannose residue by Golgi α-mannosidase I (7), and finally a second molecule of N-acetylglucosamine and a molecule of fucose are added by N-acetylglucosaminyltransferase II (8) and fucosyltransferase (9) respectively.

trans cisternae:two molecules of galactose are added by galactosyltransferase (10), followed by addition of two molecules of of sialic acid by sialyltransferase (11).


ER
1
2
3
4
P
P
Dol
cis
Golgi
5
medial
Golgi
6
7
8/9
trans
Golgi
10
11
KEY
N-acetylglucosamine
fucose
mannose
galactose
glusose
sialic acid

normal proteins or only binds to proteins which are folded incorrectly is still unknown, although recent evidence demonstrates that *in vitro* GRP78 is only found in association with malfolded proteins (Kassenbrock *et al.*, 1988).

1.1.3 The Golgi complex.

The Golgi complex consists of 3-8 flattened cisternae which are grouped together in a characteristic stack (for reviews see Rothman 1981, Farquhar 1985). One side of this stack faces the ER and is termed the *cis* side, whereas the *trans* side faces secretion granules or centrioles; cisternae which lie in between *cis* and *trans* are sometimes called medial. Newly synthesised proteins enter at the *cis* cisterna and traverse the subsequent cisternae in a unidirectional fashion.

Transfer of proteins from the ER to the Golgi is thought to be mediated by shuttling vesicles. Specialised regions of the RER known as transitional elements, which lack ribosomes, can often be seen to support many budding structures under the electron microscope (EM). It has been difficult to prove however, if these vesicles represent true intermediates in transport to the Golgi from ER, or whether they are delivering material to the ER from another subcellular compartment. The biochemical purification of these vesicles still remains elusive, although a possible candidate for the vesicles has been detected in homogenates of HepG2 cells (Lodish *et al.*, 1987). The recent development of *in vitro* systems (Haselbeck and Schekman 1986, Balch *et al.*, 1987) and semi-*in vitro* systems (Beckers *et al.*, 1987) which faithfully reproduce transport of material from ER to the Golgi complex will no doubt aid in the characterisation of these vesicles and in the identification of molecules required for transport.

Transport between adjacent cisternae of the Golgi complex is also thought to be mediated by budding and fusion of vesicles (for review see Pfeffer and Rothman 1987). Cisternae are rarely seen to come into contact with each other, and therefore

each individual cisterna represents a subcompartment of the Golgi. The pH, ionic strength, and substrate concentrations can therefore be regulated and do vary between the *cis* and *trans* sides of the stack. This heterogeneity and high degree of compartmentalisation is also reflected in the wide variety of functions performed by the Golgi complex, including: N-linked terminal glycosylation (for review see Kornfeld and Kornfeld 1985); O-linked glycosylation (for review see Kornfeld and Kornfeld 1985); sulphation (Huttner 1988); phosphorylation of lysosomal enzymes (von Figura and Hasilik 1986); and proteolytic processing of prohormones (for review see Thomas *et al.*, 1988).

The processing of core oligosaccharide chains added to the polypeptide in the ER is one of the main pieces of evidence for the functional compartmentalisation of the Golgi. Briefly, the high mannose precursor chains are usually trimmed by mannosidases and further modified by a series of glycosyltransferases. Five outer mannose residues are removed and a triantennary structure (composed of GlcNAc, galactose, and sialic acid) is constructed on the remaining core. Membrane fractionation studies indicate that the various stages of the carbohydrate processing pathway is localised to specific regions within the Golgi (see fig. 1.2), which provides a spatial and temporal mechanism for control of the sequential maturation of the newly synthesised glycoproteins.

Maturation of oligosaccharide side chains has been used as a biochemical marker of transport through the Golgi during the development of an *in vitro* system (Fries and Rothman 1980). In this system the G glycoprotein of vesicular stomatitis virus (VSV) has been used as a model protein to follow transport (Balch *et al.*, 1984a,b). Donor membranes were prepared from a VSV-infected Chinese hamster ovary (CHO) cell line that is deficient in N-acetylglucosamine transferase I activity, and is therefore incapable of adding N-acetylglucosamine to the oligosaccharide chain (see fig.1.2). The membranes were mixed with acceptor membranes prepared from uninfected wild type cells in the presence of ATP, an ATP-generating system,

cytosolic proteins, and UDP-[^{3}H]GlcNAc. Under these conditions radiolabelled GlcNAc was incorporated into G glycoprotein from the donor membranes by the enzyme located in the acceptor membranes. This is taken to represent transport of G protein from the donor Golgi to the acceptor Golgi. This transport system has also been reproduced *in vivo* : a VSV-infected CHO cell that was competent for the addition of GlcNAc but not galactose to the oligosaccharide chain was fused with a second cell that was deficient in the addition of GlcNAc but was able to add galactose. Following cell fusion VSV G glycoprotein became correctly glycosylated (Rothman *et al.*, 1984a,b).

Reconstitution of inter-cisternal protein transport will ultimately allow the identification of molecules that facilitate the process. Balch and Rothman (1985) have reported that pretreatment with N-ethylmaleimide (NEM) of "donor" and "acceptor" membranes in their *in vitro* system completely abolishes the transport reaction, suggesting a requirement for an N-ethylmaleimide sensitive factor (NSF) during secretion. Inhibition of transport does not occur however, if only "donor" or "acceptor" membranes are pretreated; and moreover, transport can be restored if donor membranes from uninfected cells (ie lacking G protein) are added to the reaction. Taken together, this implies that NSF is periphally bound to membrane, but can dissociate from this membrane under the conditions of the transport reaction and replace endogenous NSF that has been inactivated with NEM. Subsequent investigations have revealed that NSF has an approximate molecular weight of 100K and is inactivated by boiling or trypsin treatment - consistent with it being proteinaceous (Glick and Rothman 1987).

Much evidence suggests the Golgi to be the point within the secretory pathway where different classes of protein are sorted in preparation for delivery to alternative locations within the cell (for review see Farquhar 1985). The oligosaccharide moieties on all the different types of protein are terminally modified, which must mean that sorting occurs either in a *trans* Golgi cisternae or following exit from the

organelle. Griffiths and Simons (1986) propose the existence of a specialised organelle on the trans side of the Golgi termed the "trans Golgi network" (TGN, see fig.1.1), previously referred to as GERL, trans tubular network, and *trans* Golgi reticulum, in which this segregation occurs. This hypothesis is based upon the observation that, at 20°C, transport of newly synthesised viral glycoproteins from the ER to the cell surface is blocked (Matlin and Simons 1983, Saraste and Kuismanen 1984). Examination of accumulated material revealed that it was terminally glycosylated (Fuller *et al.,* 1985), and that it co-localised with an enzyme marker specific for the TGN (acid phosphatase, Griffiths *et al.,* 1985).

1.1.4 Transport of proteins to the cell surface and their release by exocytosis.

The contents of vesicles are released from the cell by an exocytotic mechanism. However, Tartakoff and Vassali (1978) have shown that cells possess either a constitutive and/or a regulated mode of vesicle release, and have thus defined cells in a similiar manner. Regulated secretory cells are specialised to release, over a short period of time, large amounts of protein at a much higher rate than the synthetic rate. This is brought about by the storage of newly synthesised material in secretory vesicles that, in the absence of stimulation, have a half life of several days, and therefore become concentrated within the cytoplasm. Cells of the endocrine system are known to concentrate secreted proteins by as much as 200-fold during their passage from the *trans* Golgi to the mature vesicle (Salpeter and Farquhar 1981). Fusion of these vesicles with the plasma membrane, and subsequent release of their contents is determined by changes in the concentration of an intracellular message, which is usually calcium. At present several investigations are directed at determining the mechanism by which this occurs (see 1.1.5). In contrast, constitutive secretory cells do not concentrate newly synthesised molecules - there is no post Golgi storage pool and the transport vesicles move rapidly from the Golgi to the plasma membrane, where fusion occurs constitutively.

Direct evidence for the existence of two types of secretion within the same cell has been obtained through studies of the endocrine cell line AtT-20. These cells naturally secrete adrenocorticotrophic hormone (ACTH), but this particular cell line also possesses an endogenous Murine leukaemia virus and therefore synthesises a viral coat protein (gp70) which is transported to the plasma membrane prior to viral budding. Gumbiner and Kelly (1982) have shown that secretory vesicles which contain newly synthesised ACTH do not contain any newly synthesised gp70 and vice-versa; and also found that only ACTH was stored within mature secretory granules and subsequently released on exposure to an external stimulus. In the absence of stimulation however, approximately 10% of the ACTH precursor (proopromelanocortin, POMC) was "leaked" from cells with kinetics indistinguishable from those of gp70. From these observations it was hypothesised that POMC is stored in secretory vesicles in the regulated pathway, processed proteolytically to mature ACTH and released only upon stimulation. In the constitutive pathway POMC could be externalised in the absence of stimulation by the same route that carries gp70 membrane protein.

Differences also exist between cells as to the sites chosen for exocytosis; cells can be catagorised as being either polarised or non-polarised depending upon the distribution of exocytotic events over the cell surface (for review see Kelly 1985). Fibroblasts are excellent examples of non-polarised secretors, with secretion of components of the extracellular matrix occuring anywhere over the entire cell surface. In contrast, endocrine and exocrine cells are examples of polarised secretory cells, localising the release of their products to a specialised domain of the plasma membrane. A secretory cell can therefore be either regulated or constitutive, and polarised or non-polarised.

1.1.5 The chromaffin cell: an example of regulated secretion.

One of the best studied regulated secretory systems is the chromaffin cell of the adrenal gland - a compound endocrine gland consisting of an inner medulla surrounded by an outer cortex. Chromaffin cells are restricted to the medulla region, and are specialised to secrete, upon stimulation, copious amounts of catecholamines (adrenaline and noradrenaline), nucleotides, and soluble proteins, all of which are packaged into secretory granules (referred to as chromaffin granules in chromaffin cells; for review see Burgoyne 1984, Winkler *et al.,* 1986) Stimulation of the adrenal medulla is by the splanchic nerve of the sympathetic nervous system, via release of acetylcholine over chromaffin cells (Elliot 1913, Feldburg 1934). It is believed however, that acetylcholine initiates a chain of events leading to a rise in intracellular calcium levels, which forms the signal for secretion (Douglas *et al.,* 1967).

Calcium may exert its effects through the ubiquitous calcium receptor protein, calmodulin (Cheung 1980), since antagonists of calmodulin (e.g the phenothiazines, Levine and Weiss 1977) or anti-calmodulin antibodies (Trifaro and Kenigsburg 1983) both perturb secretion of catecholamines from chromaffin cells (Wada *et al.,* 1983, Trifaro and Kenigsburg 1983). In fact, chromaffin granule membranes possess binding sites for calmodulin (Burgoyne and Geisow 1981), which can be classified as either calcium independent or dependent (Geisow *et al.,* 1982): calmodulin binds to polypeptides of molecular weight 22K and 25K in the presence of EGTA (a chelator of calcium) and to polypeptides of molecular weight 50K and 69K in the presence of 1mM calcium; consistent with an increase in the binding of calmodulin to calcium dependent sites following calcium influx in stimulated cells (Phillips 1974, Geisow and Burgoyne 1983) .

In addition to calmodulin, several more cytosolic proteins are recruited by the chromaffin granule during calcium stimulation (Geisow and Burgoyne 1982, Creutz

et al., 1983). Geisow and Burgoyne (1982) mixed purified chromaffin granules with an adrenal medulla extract, in the presence of micromolar calcium, and observed that proteins of molecular weight 33-37K and 70-77K became bound to granules. Binding of these proteins was reversible since they were removed by washing in EGTA buffer. Alternatively, Creutz *et al.,* (1983) immobilised chromaffin granule membranes onto a chromatography column, and then passed a cytosolic fraction through this column in the presence of 2mM calcium. Bound proteins were subsequently eluted from the column by step-wise washing in solutions of decreasing calcium concentration. In total, twenty two "chromobindins" were isolated by this method and can be classified, as shown in table 1.1, depending upon whether: interaction is via phospholipid as opposed to an integral membrane protein; whether recruitment to the membrane is ATP dependent; or whether they can be found in tissues other than the adrenal medulla. These results should be treated with caution however, since during stimulated secretion the concentration of calcium rises from a resting state of approximately 0.1μM to micromolar levels only (Baker and Knight 1978, Knight and Baker 1982), and therefore the significance of proteins which only bind to chromaffin granule membranes at calcium concentrations no lower than 40μM is uncertain.

Several of the proteins listed in table 1.1 have been further characterised. Space does not permit discussion of each individual one, although specific examples will be mentioned below. Three of the chromobindins: chromobindin XV (alternatively p70 or synhibin), chromobindin VIII (alternatively p36 or calpactin I), and chromobindin IV (alternatively p32.5 or endonexin) have all been demonstrated to cross react with antiserum to calelectrin (Geisow *et al.,* 1986). Calelectrin is a calcium binding protein, of molecular weight 34K, that was isolated from the electroplax tissue of the ray *Torpedo marmorata* (a tissue very rich in cholinergic synaptic membranes; Sudhof *at al.,* 1984, Geisow *et al.,* 1984). Geisow *et al.,* (1986) cleaved calelectrin and chromobindins VIII and IV with either trypsin or cyanogen bromide, purified the resulting peptide fragments, and sequenced those that

Table 1.1 Characteristics of the chromaffin granule binding proteins.

Protein	Mol wt	Isoelectric point	Ca^{2+a}	ATP[b]	Lipid[c]	Liver[d]
Chromobindin I	19K		0.1	-	-	+
Chromobindin II	26K	5.7	40	-	-	+
Chromobindin III	26K	6.8	0.1	-	PS, G	+/-
Chromobindin IV	34K	5.8	0.1	-	G	++
Chromobindin V	34K	5.2	40	-	PS, G	+
Chromobindin VI	35K	6.7	40	-	PS, G	+
Chromobindin VII	36K	5.1	40	-	PS, G	+/-
Chromobindin VIII	37K	7.9	0.1	-	G	+
Chromobindin IX	38K	6.9	40	-	PS, G	+
Chromobindin X	46K	5.6	40	-	PS, G	+
Chromobindin XI	47K	7.0	0.1	-	PS, G	+
Chromobindin XII	53K	6.8	40	+	-	+
Chromobindin XIII	55K	7.8	40	+	-	+
Chromobindin XIV	56K	5.9	40	+	-	+
Chromobindin XV	56.5K	6.2	40	+	-	+
Chromobindin XVI	57K	7.0	40	+	-	+
Chromobindin XVII	57.5K	5.7	0.1	-	PS, G	+
Chromobindin XVIII	58K	6.0	40	+	-	+
Chromobindin XIX	59K	7.0	40	+	-	+
Chromobindin XX	67K	6.1	0.1	-	PS, G	++
Chromobindin XXI	66K	5.6	40	-	PS, G	+
Chromobindin XXII			0.1	-	PS, G	-

This table shows some characteristics of the twenty three chromobindins described by Creutz *et al.*, (1988). See text for details.

a. Calcium concentration at which the majority of each species elutes from the affinity column. 40, 40μM; 0.1, 0.1μM.

b. Dependence on ATP for binding. +, ATP required. -, ATP not required.

c. Ability to bind to liposome column. -, no binding; PS, binding to phosphatidylserine; G, binding to granule lipids.

d. Indicates whether the protein was obtained from liver cytosol. +, comparable yield to that from the medulla; ++, particulary abundant in fractions obtained from the liver; +/- obtained in reduced yield relative to medullary fractions.

reacted with anti-calectrin antibodies. All fragments contained a consensus sequence of seventeen residues, that occurs at least twice in each protein. The functional significance of this repeat sequence is unknown at present, although since all the proteins bind calcium but do not possess sequences previously correlated with calcium binding (the EF hand, Kretsinger 1980) it is tempting to speculate that it represents an alternative calcium binding domain. The discovery of this immunological relationship has led to this class of proteins being given the generic name "annexins" (for review see Geisow *et al.,* 1987). Other functional characteristics assigned to proteins in this group include the potential to bind calcium independent of membrane, and their ability to promote aggregation of chromaffin granules in the presence of calcium. The ability of annexins to bind membrane is not influenced by pretreatment of membranes with trypsin, indicating that the interaction may be with phospholipid (Geisow *et al.,* 1987).

During stimulation, chromaffin granules move from intracellular sites to the plasma membrane. This movememt could be diffusion but the rapidity of secretion has led to the speculation that granules associate with the cytoskeletal system that, by analogy to recent findings concerning the movement of vesicles during fast axonal transport, is capable of generating motive force (for review see Vale 1987). In support of this hypothesis, actin has been found in association with chromaffin granule membranes, and may function as assembly sites for actin microfilaments (Burridge and Phillips 1975, Fowler and Pollard 1982, Wilkins and Lin 1981). The physiological significance of this is uncertain however, since this is maximal at 0.1μM calcium and is inhibited at the higher calcium concentrations experienced during secretion. Also, pretreatment of chromaffin cells with cytocholasin B, which promotes dissassembly of actin filaments (Schneider *et al.,* 1981, Knight and Baker 1982), has only marginal effects on secretion; whereas a stabiliser of actin filaments, phalloidin, inhibits secretion (Lelkes *et al.,* 1986). These characteristics are consistent with a model in which calcium influx causes generalised breakdown of actin filaments and subsequent release of granules from the actin network; in this

view the cytoskeleton prevents exocytosis. In support of this, secretion is inhibited when antibodies directed against fodrin are introduced into permeabilised chromaffin cells (Perrin *et al.,* 1987). Fodrin, the non-erythrocyte counterpart of spectrin, is found in the cytoplasm of all cells, but is usually localised to the under surface of the plasma membrane, and membranes of secretory granules. Two previous investigations assist in the interpretation of this result: Perrin and Aunis (1985) reported that fodrin can bind to actin filaments, and proposed one of its functions to be anchoring filaments to the plasma membrane; and Siman *et al.,* (1985) demonstrated that fodrin is highly susceptible to cleavage by a calcium activated protease, which can be prevented by incubation of chromaffin cell membranes with anti-fodrin antiserum. Thus one process that may occur during stimulation of chromaffin cells is calcium activated proteolysis of fodrin, ultimately leading to breakdown of the peripheral cytoskeletal network, allowing granules access to exocytotic sites.

In relation to this, one of the proteins of molecular weight 70K observed to bind to chromaffin granules in the presence of calcium (see above) has been identified by immunological criteria to be caldesmon (Geisow and Burgoyne 1982, Burgoyne *et al.*, 1986) . This protein was originally identified in chicken smooth muscle as a calmodulin regulated actin binding protein, but has since been found in a range of tissues and cell types. In similiar fashion to fodrin, caldesmon is localised to the periphery of the cell and has been implicated in the control of actin assembly. In the absence of calcium, caldesmon stimulates assembly of actin and cross-linking of actin filaments; both of these characteristics are inhibited by levels of calcium similiar to those observed during secretion.

Two new concepts have emerged from investigations of the chromaffin cell: in the absence of stimulation, secretory granules are prevented from interacting with exocytotic sites on the plasma membrane, by a peripheral meshwork of the cytoskeleton; and that secretory granules in unstimulated cells may not possess all

the vital functions required for membrane fusion - these proteins are assembled onto the granule during stimulation. Further investigation is required to ascertain if these mechanisms are specific to the chromaffin cell, or regulated secretory cells in general; but since several of the proteins described above have been identified in numerous cell types, this system could be present in all cells that have regulated secretory pathways.

1.1.6 The sorting problem.

The vast assortment of proteins that are present within the lumen and membrane of the ER present the cell with a fundamental problem. In order to maintain the integrity of the secretory pathway the cell must possess an efficient mechanism by which it can recognise different classes of protein, and successfully segregate them to their intended intracellular location.

Figure 1.3 represents the model proposed by Pfeffer and Rothman (1987) to explain how the cell accomplishes this task. Transport between organelles is mediated by budding and fusion of vesicles, and is unidirectional and energy dependent. Proteins which are to establish an intracellular location within the pathway, ie luminal and membrane proteins of the ER, Golgi, lysosomes, and secretory granules must possess a targetting signal which is recognised by the cell and used to restrict their distribution. Further proteins which do not possess a targetting signal will successfully traverse the secretory pathway, eventually being released by constitutive exocytosis.

An alternative model has been presented by Lodish and coworkers (1983). This hypothesis was formulated in response to their observation that different proteins are secreted at different rates: in hepatoma (HepG2) cells at 32°C the half time for release of albumin and α1-antitrypsin is 45 min, C3 complement and α1-antichymotrypsin 85-90 min, and transferrin 140 min. Further investigation

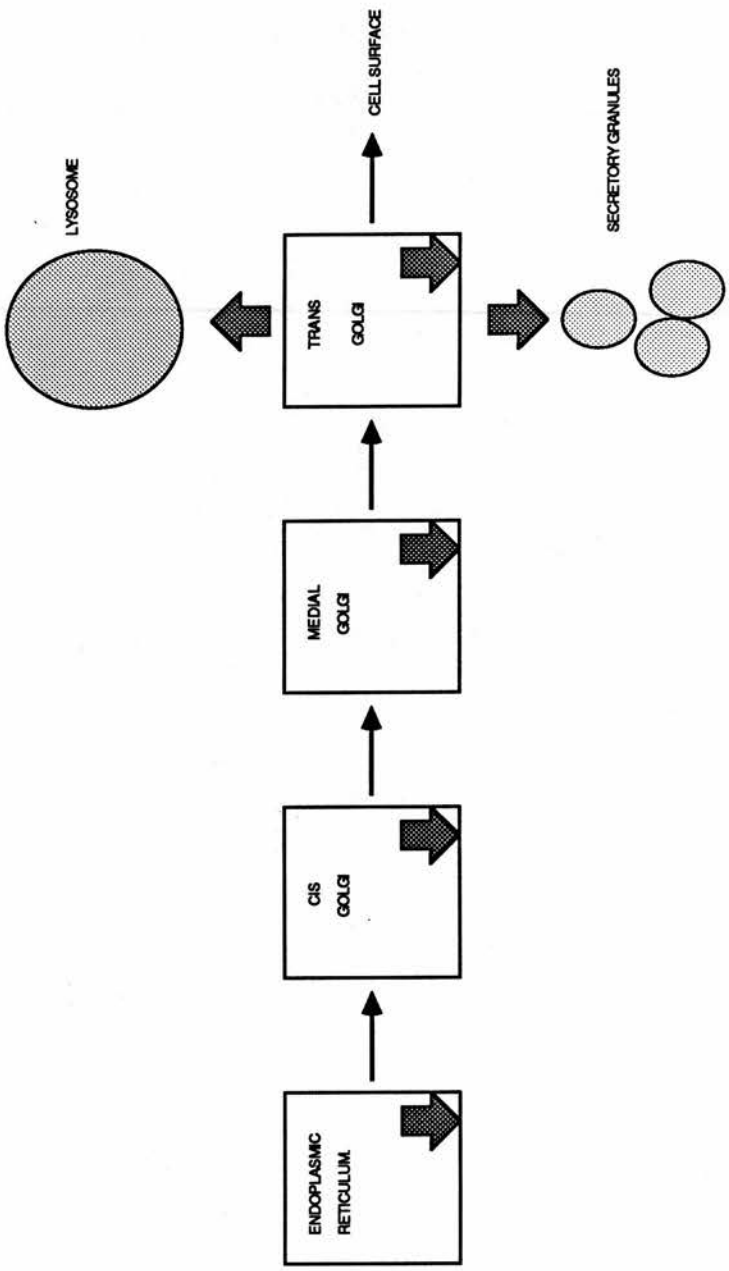
revealed that the rate-limiting step in the secretion of these proteins was their release from the ER. The authors proposed the existence of specific receptors within the ER to which secretory proteins must bind prior to transport to the Golgi. In this view proteins which have a high affinity for such receptors would bind rapidly and spend only a short time in the ER (e.g albumin and α_1-antitrypsin), whereas slow moving proteins such as transferrin would have low affinity and would move out of the ER only at the speed of bulk flow.

Wieland *et al.,* (1987) have presented evidence which supports the former model of "secretion by default". They used the synthetic acyl tripeptide N acyl-Asn-Thr-Thr (where N is a fatty acid with varying chain length of 2-10 carbon atoms), which is capable of diffusion across membranes. Following diffusion into the ER however, the polypeptide becomes glycosylated and can only then be released from the cell via the secretory pathway. Since the tripeptide is so small it can be assumed to lack any sorting signal and will therefore be conveyed by bulk flow. The observed kinetics of release of the glycosylated tripeptide were at least equal to, in many cases more rapid, than the secretion of any known protein suggesting that bulk flow is sufficiently rapid to carry secretory proteins to the cell surface. Differences in the length of time proteins remain within the ER, in this view, is probably attributable to the time required to attain a suitable conformation prior to movement from the ER through the secretory pathway (Wieland *et al.,* 1987).

Further evidence in support of the "secretion by default" model has been obtained by the identification of specific sorting signals on two different classes of protein. Three resident proteins of the ER lumen - Bip/GRP78, GRP94, and protein disulphide isomerase (PDI) all have the same tetrapeptide sequence (KDEL) at their carboxy-terminus. The hypothesis that this represents a true retention signal was tested by either deleting the tetrapeptide or by adding further amino acids to the carboxy-terminus of GRP78. Both treatments resulted in the secretion of GRP78 (Munro and Pelham 1987). The tetrapeptide sequence is all that is needed for

Figure 1.3 **Signal mediated retention of proteins in the secretory pathway.**

This model has been proposed by Pfeffer and Rothman (1987). Transport from the ER to the cell surface is accomplished in a number of stages (↓) which are signal independent and represent bulk flow. Alternatively, proteins that are located in specific compartments of the secretory pathway, or proteins that are diverted to the lysosome or secretory granules must possess a sorting signal that is recognised by some mechanism, which operates to restrict the flow of these proteins along the pathway; ⇩ represents those transport steps that are signal mediated.


ENDOPLASMIC RETICULUM
CIS GOLGI
MEDIAL GOLGI
TRANS GOLGI
LYSOSOME
CELL SURFACE
SECRETORY GRANULES

retention since when this is added to the carboxy-terminus of lysozyme, a secreted protein under normal conditions, it becomes localised to the ER (Munro and Pelham 1987). Whether the signal acts by anchoring the protein to a second protein in the ER, or as a signal for recapture following escape to the Golgi is still unknown although recent evidence supports the latter (Pelham 1988, Cerriotti and Coleman 1988).

One signal which is used to segregate soluble proteins to the lysosome is also known (for review see von Figura and Hasilik 1986). Upon arrival at the *cis* Golgi, lysosomal hydrolases are modified by the combined action of a GlcNAc-phosphotransferase and a GlcNAc-phosphodiesterase, which have the combined effect of phosphorylating the sixth carbon position of certain mannose (M-6-P) residues within the oligosaccharide side chains. This modification serves as an address signal and is recognised by one of two M-6-P receptors of molecular weight 215K and 46K. Conclusive evidence of where the hydrolase-receptor complex is formed within the Golgi is still lacking.

1.2 ENDOCYTOSIS IN THE EUKARYOTIC CELL.

As described above, it is generally accepted that movement of proteins between different compartments of the secretory apparatus is mediated by budding and fusion of vesicles. Major insights into the mechanisms of intracellular vesicle movement have been gained from investigations of endocytosis.

Endocytosis is almost certainly ubiquitous amongst eukaryotic cells. In general, the process is one by which cells ingest extracellular materials by trapping them within inward foldings of the plasma membrane which subsequently pinch off to form intracellular vesicles. Two types of endocytosis have been described, namely: fluid phase endocytosis, which involves material in the fluid phase becoming trapped in the invagination during internalisation; and receptor-mediated endocytosis, during

which there is an association of the intended ligand with a specific receptor on the plasma membrane (for reviews see Goldstein *et al.,* 1979, 1985). Both of these routes are thought to be involved in several processes. Endocytosis provides a mechanism by which animal cells obtain many micronutrients including iron, cholesterol, and vitamin B12 (Hemmaplardh and Morgan 1976, Newmark *et al.,* 1970, Anderson *et al.,* 1977). It also provides a route into the cell for many hormones, which enter the cell in hormone-receptor complexes (Goldstein *et al.,* 1976). In the context of this thesis, endocytosis performs a more relevant role in what is loosely termed "membrane conservation". Many cells secrete high levels of proteins by fusion of secretory vesicles with the plasma membrane, yet the cells are not observed to increase in size. Therefore the cell must possess a mechanism by which an amount of membrane can be recaptured which is equivalent to that supplied during secretion. Endocytosis is thought to fulfill this function. In addition to these vital roles, the endocytic pathway is subverted and used as a method of entry into the cell for certain viruses (e.g influenza virus, Helenius *et al.,* 1980) and toxins (e.g diphtheria, Draper and Simon 1980).

Our understanding of the mechanism of endocytosis has largely been acquired through studies, in mammalian systems, of the internalisation of individual proteins, including epidermal growth factor (EGF), asialoglycoproteins (ASGP), and transferrin; and small particles, e.g low density lipoprotein (LDL) - all of which are taken up via a receptor-mediated pathway. The first event which must occur during receptor-mediated endocytosis is the formation of a receptor-ligand complex at the cell surface, after which such complexes become concentrated in specialised regions of the plasma membrane termed coated pits (for review see Bretscher and Pearse 1984). For the majority of ligands, e.g LDL (Anderson *et al.,* 1982, Basu *et al.,* 1981), transferrin (Hopkins and Towbridge 1983, Hopkins 1985), α-2 macroglobulin (Hopkins 1982, Via *et al.,* 1982), ASGP (Wall *et al.,* 1980, Berg *et al.,* 1983), and insulin (Krupp and Lane 1982), the receptors are randomly distributed over the cell surface but are able to spontaneously move into coated pits

an enter cells continuously. In the case of EGF however, its receptors are randomly distributed over the cell surface but they are unable to enter coated pits unless they are in complex with ligand (Schlessinger 1980, Dunn and Hubbard 1984). Certain gross features are common to the various receptors known to enter coated pits. Each receptor comprises a large extracellular portion containing the ligand binding site, a single helix spanning the membrane and a relatively small cytoplasmic portion. A recent experiment demonstrates that the cytoplasmic tail may be of fundamental importance during internalisation of the protein (Roth *et al.*, 1986). The study investigated the properties of two viral glycoproteins: the G glycoprotein of VSV which cycles from the plasma membrane, and the haemagglutinin (HA) of influenza virus which does not. Using genetic engineering the transmembrane and cytoplasmic tail domains of HA were replaced with those of G protein. The resulting hybrid protein was able to enter coated pits on the endocytic pathway. Despite the significance of this result however, no common feature amongst the cytoplasmic tails of different proteins has been recognised.

Once a full complement of material has been recruited into the coated pit, there is a membrane fusion event at the neck of the invagination releasing a coated vesicle into the cytoplasm. Within a very short period of time the vesicles are observed to lose their coats, a reaction catalysed by a 70K "uncoating ATPase" (Chappell *et al.*, 1986, for review see Rothman and Schmid 1986). This event exposes the membrane of the vesicles in preparation for fusion with membrane at its next destination. Naked vesicles fuse with an organelle termed the endosome. This is almost certainly the location at which internalised material is sorted prior to delivery to different compartments within the cell (for review see Hopkins 1983). The endosome has an acidic internal pH which is maintained by a membrane bound ATPase (Tycko and Maxfield 1982, Golloway *et al.*, 1983). All internalised material becomes exposed to this acidic enviroment but the subsequent response that is initiated is specific to the receptor-ligand complex (for review see Goldstein *et al.*, 1985 and references therein). In the cases of LDL and ASGP the ligands dissociate from their receptors

and are ultimately delivered to the lysosomes, whilst the receptor molecules are recycled to the cell surface to participate in further rounds of endocytosis. Alternatively, both the ligand and receptor may be recycled back to the cell surface thereby conserving both molecules - transferrin and its receptor follow such a pathway. Where it is necessary for the cell to degrade both the ligand and receptor both molecules are delivered to the lysosome: EGF is an excellent example of this.

Several biochemical investigations have produced results which indicate that the secretory and endocytic pathways can pass through common organelles, and are therefore interconnected. Snider and Rogers (1985) observed that a fraction of internalised transferrin receptor (TfR) molecules cycle through the Golgi complex prior to being re-exported to the cell surface. Using the enzyme neuraminidase, at 0°C, the workers were able to remove all sialic acid residues from exposed TfR molecules on the cell surface; on return to 37°C in the presence of fresh growth medium resialylated TfR was observed on the cell surface after 30min. This must mean that some internalised TfR passed through the *trans* Golgi cisternae in which sialyltransferase is located; in fact, further observations suggest that TfR can be recycled as far as *cis* and medial cisternae (Snider and Rogers 1986, Woods *et al.,* 1986). The physiological significance of this route remains to be determined, although it has been suggested that molecules which pass down this pathway are in need of repair (Farquhar 1981). In principal any biosynthetic event that normally occurs in the Golgi complex including O-linked glycosylation, late stages of N-linked glycosylation, sulphation, and proteolytic processing could occur in transit during recycling as well as during the initial biosynthetic transport of newly synthesised proteins through the Golgi.

1.2.1 Structure and composition of coated vesicles.

Coated vesicles were originally isolated from pig brain (Pearse 1975, and for reviews see Pearse 1987, Pearse and Crowther 1987), but have since been isolated

from numerous mammalian tissues (Pearse 1976), plant tissues (see Pearse and Crowther 1987), and yeast (Mueller and Branton 1984). Examination of purified material by SDS-PAGE reveals that their composition is very similiar, with the major constituent invariably being a polypeptide of approximately molecular weight 180K, termed clathrin (Pearse 1976); but several additional species of molecular weights 100-120K, 50-55K, and 32-36K are also present. Clathrin is a non-glycosylated polypeptide which can account for up to 70% of the protein in a vesicle coat, and is thought to play a fundamental role in the formation of coated pits and vesicles. From its sensitivity to proteases and response to other mild treatments (e.g high pH washing) it is known to reside on the cytoplasmic face of vesicles (see Pearse 1987). When clathrin is dissociated from vesicles by mild treatment it is more often than not found in a characteristic "triskelion" (Ungewickell and Branton 1981). Each leg of the triskelion is approximately 45nm long and is comprised of a molecule of clathrin in association with a protein of approximate molecular weight 35K; because of this association the 180K molecular weight protein is called the clathrin heavy chain and the 35K molecular weight protein the clathrin light chain.

Coated vesicles are heterogeneous in size, ranging from 50nm-150nm diameter, but are remarkably similiar in shape. The vesicles have a surface lattice of hexagons and pentagons, most are built from 12 pentagons in combination with a variable number of hexagons, which determines the size of the vesicle (Heuser 1980, Nermut 1982). Depending upon the conditions of extraction, clathrin triskelions will spontaneously assemble to form clathrin "cages", which resemble the polyhedral structure of coated vesicles seen *in vivo* (Ungewickell and Branton 1982). This *in vitro* reaction, and the association of clathrin with developing coated pits *in vivo*, is promoted by a family of proteins, of approximate molecular weight 100-110K, which are also found in the coat of vesicles (Zaremba and Keen 1983, 1985). Vesicle coats from bovine brain contain six proteins in this family which are usually present in a ratio of approximately one 100K polypeptide per clathrin trimer. Pearse (1985) observed that a receptor protein normally included in coated pits

associated the 100-110Kd accessory proteins *in vitro* to produce small spherical structures of diameter 30-100nm, with an apparent stoichiometry of approximately one receptor molecule per 100K polypeptide. When clathrin was included in the assembly mixture these cores became encapsulated in clathrin cages. These experiments provide a clue about the mechanism of coated pit and vesicle formation: the 100-110K molecular weight proteins are thought to interact with the membrane of the coated pit, perhaps by recognising the cytoplasmic tails of receptor proteins, which in turn promotes crystallisation of clathrin on the membrane to drive development of the vesicle. As there is a variety of 100-110K molecular weight polypeptides the possibilty exists that the different molecules tend to occur on different membranes in cells and that individual ones bind to specific groups of membrane proteins in their respective membranes (Pearse 1985).

The cytoskeleton has been proposed to mediate intracellular movement of coated vesicles throughout the cell (Lacy and Malaisse 1973, Reaven and Reaven 1980). This is based upon morphological findings which suggest an association between coated structures, microtubules, and actin filaments. In support of this hypothesis two vesicle coat proteins of molecular weight 54K and 56K have been identified as α-tubulin and ß-tubulin respectively by immunological criteria (Pfeffer *et al.,* 1983, Kelly *et al.,* 1983). Association of coated membrane with cytoskeletal fibres could also facilitate the formation of coated vesicles since contraction of such fibres would effectively pull the coated membrane into the cytoplasm, thereby aiding development of the coated pit.

1.2.2 Coated pits and vesicles in secretion.

Rothman and Fine (1980) presented evidence to support the existence of clathrin coated vesicles which convey newly synthesised proteins from the ER to the Golgi, and from the Golgi to the plasma membrane. Recent investigations however, have refuted these observations; but moreover, indicate that clathrin does not play an important

role in the movement of membrane during secretion. Morphological examination of secretory cells reveal that secretory vesicles, coated with clathrin, can only be detected budding from the *trans* Golgi network (Griffiths *et al.*, 1985, Orci *et al.*, 1985). It is this compartment that holds centre stage in the sorting of proteins to their alternative locations within the cell (Griffiths and Simons 1986). It has been difficult to prove however, if these vesicles are delivering material to the organelle from a different compartment, or if they are true carriers of biosynthetic material.

Compelling evidence is now available that argues against clathrin coated vesicle participation in constitutive secretion. Using immunolabelling techniques, Griffiths *et al.*, (1985) and others (Wehland *et al.*,1982, Orci *et al.*, 1986) failed to detect VSV G glycoprotein in clathrin coated vesicles budding from the Golgi. In support of these morphological studies, introduction of monoclonal antibodies, directed against the clathrin heavy chain, into cells had no detrimental effect on the constitutive release of proteins, but did severly inhibit endocytosis (Doxsey *et al.*, 1987)

In an attempt to re-examine the transport of newly synthesised proteins through the cisternae of the Golgi, Orci *et al.*, (1986) observed a novel class of coated vesicle. Using the *in vitro* system of Rothman and colleagues (see section 1.1.3), donor membranes were seen to support many budding structures and complete vesicles when incubated with cytosolic proteins in the presence of ATP. Both of these structures appeared to possess cytoplasmic coats which were distinguishable from clathrin coats: they did not adopt the characteristic polygonal configuration, and the coats did not react with antibodies directed against clathrin heavy chain. Confirmation that these vesicles act as biosynthetic carriers was obtained by localising VSV G glycoprotein, by immunogold electron microscopy, to within developing buds and vesicles that had these characteristic coats.

The role of clathrin in regulated secretion is less clear. Mouse AtT-20 cells exhibit both constitutive and regulated secretion (Gumbiner and Kelly 1981, 1982);

approximately 80% of the ACTH the cells produce is directed into the regulated secretory pathway, being stored in secretory granules prior to release. Mature secretory granules have never been observed to be coated or to obtain a coat during stimulated secretion. However, newly synthesised ACTH can be detected in clathrin coated vesicles budding from late Golgi cisternae (Tooze and Tooze 1986). It is proposed that clathrin coated vesicles are intermediates in the formation of secretory granules, which shuttle between the TGN and immature secretory granules, delivering biosynthetic material and removing material that has been incorrectly sorted to the granule (Tooze and Tooze 1986).

Coated vesicles have been isolated from yeast cells (Mueller and Branton 1984) and clathrin triskelions purified. Examination of purified triskelions by electron microscopy revealed that they are essentially identical to their mammalian counterparts. The identification of clathrin in yeast allows powerful genetic analyses to be undertaken, and the importance of clathrin during secretion to be determined. Two groups have raised antibodies against purified clathrin triskelions, and used these to isolate the gene encoding clathrin heavy chain (*CHC1*) (Payne and Schekman 1985, Lemon and Jones 1987). Both groups chose to test the importance of clathrin in yeast metabolism by replacing the genomic *CHC1* gene with a disrupted deleted mutant *chc1* gene. Unfortunately the results obtained are contradictory: Payne and Schekman (1985) reported that cells harbouring the mutant allele are viable, but their growth is severly retarded and the cells are full of autophagic vacuoles, diagnostic of sick cells. Further examination of these cells revealed that invertase is secreted into the periplasm at rates comparible to wild type cells. In contrast Lemon and Jones (1987) reported that strains which do not possess a functional *CHC1* gene are incapable of growth, and have argued that viability of the strains studied by Payne and Schekman (1985) is due to the presence of a suppressor. Further investigation is clearly required.

1.3 ANALYSIS OF THE PROTEIN SECRETION PATHWAY IN *SACCHAROMYCES CEREVISIAE*.

Investigations into the secretion pathways of higher eukaryotes have yielded an immense amount of information about the gross features of secretory organelles, and the structure and covalent modifications of molecules that are transported through these cellular organelles. It has been much more difficult however, to define cellular functions involved in protein transport, and this difficulty has been fundamental in provoking investigations into the secretory pathway of yeast. Many of the components required for protein transport can be identified using a genetic approach, to which *S. cerevisiae* is particulary amenable. In conjunction with the recent revolution in yeast recombinant DNA methodology, this makes it an excellent system in which to study secretion (Struhl 1983).

1.3.1 The isolation and characterisation of non-secretory (*sec*) mutants.

Strains of yeast that are deficient in the secretion of proteins were isolated from cultures that had been subjected to chemical mutagenesis. Wild type yeast cells were treated with ethyl methanesulfonate or nitrous acid to induce mutation, and then tested for the ability to grow at 37°C; those strains that failed to grow were screened for the ability to secrete acid phosphatase. From this initial mutant hunt only two strains that are conditionally defective in protein secretion, designated *sec1-1* and *sec2-2*, were isolated (Novick and Schekman 1979).

Further investigation of the phenotype of *sec1-1* mutant cells revealed that at 37°C secretion of invertase and the incorporation of sulphate permease into the plasma membrane were also blocked, which correlated with an intracellular accumulation of these proteins. Much of the accumulated material however, was released from the cell on the return to the permissive temperature of 24°C, even in the presence of

cycloheximide, indicating that the *sec1-1* mutation is reversible. At the non-permissive temperature, *sec1-1* and *sec2-1* mutant cells cease to divide, but in contrast to mutants of cell division (*cdc*, Hartwell *et al.*, 1973) they do not accumulate at a particular stage of the cell cycle. Biosynthesis of protein and phospholipid continues in *sec1-1* mutant cells at 37°C for at least 3 hours, which in conjunction with the failure to increase net cell surface area leads to an increase in cell density. Henry *et al.*, (1977) observed a similiar situation during inositol starvation of an auxotrophic strain, and demonstrated that under these conditions starved cells could be separated completely from normal cells on a Ludox density gradient. Using the same techniques Novick *et al.*, (1980) observed that the 5% increase in cell density of *sec1-1* cells at 37°C allowed mutant cells to be separated completely from a 100-fold larger population of wild type cells. This characteristic proved invaluable since it provided a more efficient protocol for the isolation of further *sec* mutants.

Mutagenised cultures were grown for several generations at 24°C and then transferred to 37°C for 3 hours. Cells were then sedimented on a Ludox density gradient, and the densest 1-2% of cells harvested. Mutants that were temperature sensitive for growth were isolated from this population and screened for the ability to secrete acid phosphatase and invertase at 24°C and 37°C. Conditional secretion mutants were isolated at a frequency of 15%, a total of 485 being identified (Novick *et al.*,1980). Two distinct classes of secretion mutant were identified amongst these, dependent upon their characteristics at 37°C: class A *sec* mutants (187 in total) showed an intracellular accumulation of active invertase at the non-permissive temperature; whereas class B *sec* mutants showed no accumulation of active invertase, despite protein synthesis proceeding at a high rate at 37°C.

Recent investigation of class B *sec* mutants has determined that these strains are incapable of translocating secreted proteins across the membrane of the ER; nascent polypeptides either accumulate in the cytoplasm or become lodged in the ER

membrane. Since membrane translocation is not a central theme of this thesis I shall not present further data on these mutants, but for more information the reader is directed to Ferro-Novick *et al.,* (1984a,b), Bernstein *et al.,* (1985), Hibs and Meyer (1988), and Kepes and Schekman (1988).

Using standard genetic techniques the 187 class A *sec* mutants were arranged into 23 complementation groups (see table 1.2, Novick *et al.,* 1980). A representative allele from each group was chosen and its phenotype characterised in more detail. All *sec* mutations were recessive in heterozygous diploids, and all exhibited phenotypic traits similiar to *sec1-1*, ie. at 37°C: a cessation in the secretion of acid phosphatase and invertase, accompanied by an intracellular accumulation of these enzymes; failure to incorporate sulphate permease into the plasma membrane; and prevention of bud emergence, leading to an increase in cellular density; all of which are thermoreversible.

Thermoreversible accumulation of intracellular acid phosphatase and invertase, and the reduced incorporation of sulphate permease suggested that the *sec* mutants might accumulate an organelle of the secretory apparatus. This hypothesis was confirmed by EM analysis of thin sections of yeast cells (Novick *et al.,* 1980). Wild type cells grown at 26°C or 37°C, or *sec* mutants grown at 25°C displayed occasional enrichment of vesicles in the bud, or short thin tubules of ER in close proximity to the inner surface of the plasma membrane. Such cytological aberrations were much more pronounced in mutant cells grown at 37°C, and indeed the mutants were grouped in relation to which organelle they accumulated (see fig. 1.2). Novick *et al.,* (1980) describe these organelles as being "intermediates in the secretory pathway whose soluble contents are destined for secretion by exocytosis, and membranes for incorporation into the plasma membrane by fusion". The most common class, with 10 members, accumulated membrane enclosed vesicles of diameter 80-100nm, which in contrast to wild type cells are not localised to the bud - these are proposed to represent secretory vesicles. The second class, with 9 representatives, developed

a more extensive network of ER than that seen in wild type cells. This was often seen to line the inner surface of the plasma membrane and wind through the cytoplasm where multiple connections with the nuclear membrane became visible. A subset of mutant strains within this group (*sec17*, *sec18*, and *sec23*) also produced small 40nm vesicles which were often arranged in patches in the cytoplasm. Finally, the third class of mutants accumulated an organelle with no obvious counterpart in higher eukaryotic cells. This structure, termed the Berkeley body (Bb), varies in form but seems to consist of two curved membranes with an electron transparent lumen, is thought to represent the Golgi complex, perhaps fragmentated during incubation at the restrictive temperature (Novick *et al.*, 1980). Surprisingly, this class of mutants has only 2 representatives (*sec7* and *sec14*); if the complexity of the Golgi in *S. cerevisiae* is similiar to that in mammalian cells this is a clear underestimate of the functions required to maintain the integrity of the organelle. Finally, two mutants could not be placed into the above classes: strains harbouring mutations in the *SEC19* gene accumulated all three types of organelle at 37°C; whereas *sec11* mutant cells did not accumulate any organelles under restrictive conditions.

Novick *et al.*, (1981) suggested that the organelles accumulated in *sec* mutants at 37°C represent distinct stages in the secretory pathway. They further postulated that the secretory pathway is linear, and that double *sec* mutants constructed from single *sec* mutants that block at phenotypically distinguishable stages would accumulate the organelle corresponding to the earliest block. The possibility that the gene products acted in independent pathways which contributed to the same process would be indicated by the appearance of both phenotypes. As predicted, double mutants carrying the *sec18-1* mutation (an ER block) with the *sec7-1* (Golgi) or *sec1-1* (SV) mutations all exhibited the *sec18-1* phenotype at 37°C. Also, mutations from all ten complementation groups that show accumulation of 80-100nm SV were combined with the *sec7-1* mutation, and in each case the mutant

Table 1.2 The class A *sec* mutants.

sec mutation	Number of isolates	Organelle accumulated at 37°C.
sec1	12	SV
sec2	69	SV
sec3	3	SV
sec4	9	SV
sec5	26	SV
sec6	6	SV
sec7	4	Berkeley bodies
sec8	10	SV
sec9	7	SV
sec10	3	SV
sec11	12	No organelle accumulated
sec12	4	ER
sec13	4	ER
sec14	4	Berkeley bodies
sec15	2	SV
sec16	2	ER
sec17	1	ER, small vesicles
sec18	2	ER, small vesicles
sec19	1	ER, Berkely bodies, and SV
sec21	1	ER
sec22	4	ER, small vesicles
sec23	1	ER

Twenty three complementation groups have been identified by Novick *et al.*, (1980). Some of these groups have many representatives but others have only one; this distribution suggests that there are other *sec* A complementation groups that have not been discovered.

accumulated Bb's at 37°C. Thus, the ER accumulating phenotype is epistatic to the Golgi and SV accumulating phenotypes, and a Golgi accumulating mutant is epistatic to all the vesicles accumulating mutants. This analysis however, could hide the possibility that some gene products could be required at all stages in the secretory pathway.

1.3.2. Biochemical investigation of the yeast secretory pathway.

The development of a secretion model, derived from genetic studies, has greatly facilitated biochemical investigations of the secretory pathway in *S. cerevisiae*. Prior to the isolation of the *sec* mutants such studies were problematical due to the low level of secretory organelles in the cell and the rapid transit time of proteins through the pathway, e.g invertase takes only 5min or less to undergo complete maturation and secretion - making the isolation of intracellular precursors intrinsically difficult (Novick *et al.*, 1981). The *sec* mutants however, accumulate organelles of the secretory apparatus and secreted proteins at distinct stages in the pathway, permitting the detection of intermediates in the biogenesis of certain proteins, and the correlation between the appearance of these species and passage through particular subcompartments. In this section I shall review studies of this nature undertaken on invertase (a secreted periplasmic protein), α-factor (an exported protein), and carboxypeptidase Y (a vacuolar enzyme); similiar experiments have also been used to study the biogenesis of killer toxin (an exported protein, Bussey *et al.*, 1983). Referring to these studies I shall compare glycoprotein maturation in yeast and mammalian cells, and also emphasize the invaluable role which *sec* mutants can play in elucidating the biogenesis of a particular protein.

Secretion of invertase.

Invertase is a secreted enzyme, which remains associated with the cell wall. The polypeptide is rapidly transported to the cell surface, with a half time of approximately 5min, during which 9-10 N-linked oligosaccharides are added. Esmon and colleagues (1981) have monitored maturation of the enzyme in several *sec* mutants, and confirmed that N-linked oligosaccharides are assembled onto yeast glycoproteins in at least two stages.

In yeast, N-linked glycosylation is initiated in similiar fashion to that in mammalian cells: a core oligosaccharide is transferred from a lipid donor to the asparagine residue, in the sequence Asn-X-Ser/Thr, during translocation into the lumen of the ER (for review see Tanner and Lehle 1987). The lipid donor is charged with an immature oligosaccharide unit on the cytoplasmic face of the ER membrane, and is then translocated into the lumen of the ER where its assembly is completed (Lehle 1980). Transfer of core units to the polypeptide occurs co-translationally, and as in mammalian cells, these are trimmed prior to exit from the ER by removal of 1 mannose and 3 glucose residues (Byrd *et al.,* 1982, Esmon *et al.,* 1984). No extensive modification of core oligosaccharides occurs in yeast. Instead, many core units are elongated during transit through the Golgi complex, to produce a structure referred to as the outer chain, consisting of an α1-6 linked polymannose backbone to which numerous mannoligomers (2-4 mannose residues in length) become attached by α1-3 linkages (for review see Ballou 1982).

Analysis of invertase molecules in *sec* mutants at 37°C confirms that addition of oligosaccharides to the polypeptide is initiated in the ER, and completed during passage through the Golgi (Esmon *et al.,* 1981). Invertase accumulated in a *sec18-1* (ER) strain contains oligosaccharide chains of the structure $Man_8GlcNAc_2$ (Esmon *et al.,* 1984); addition of outer chains is not detected until invertase molecules reach the Golgi as defined by the *sec7-1* mutant.

Secretion of the mating pheromone - alpha factor.

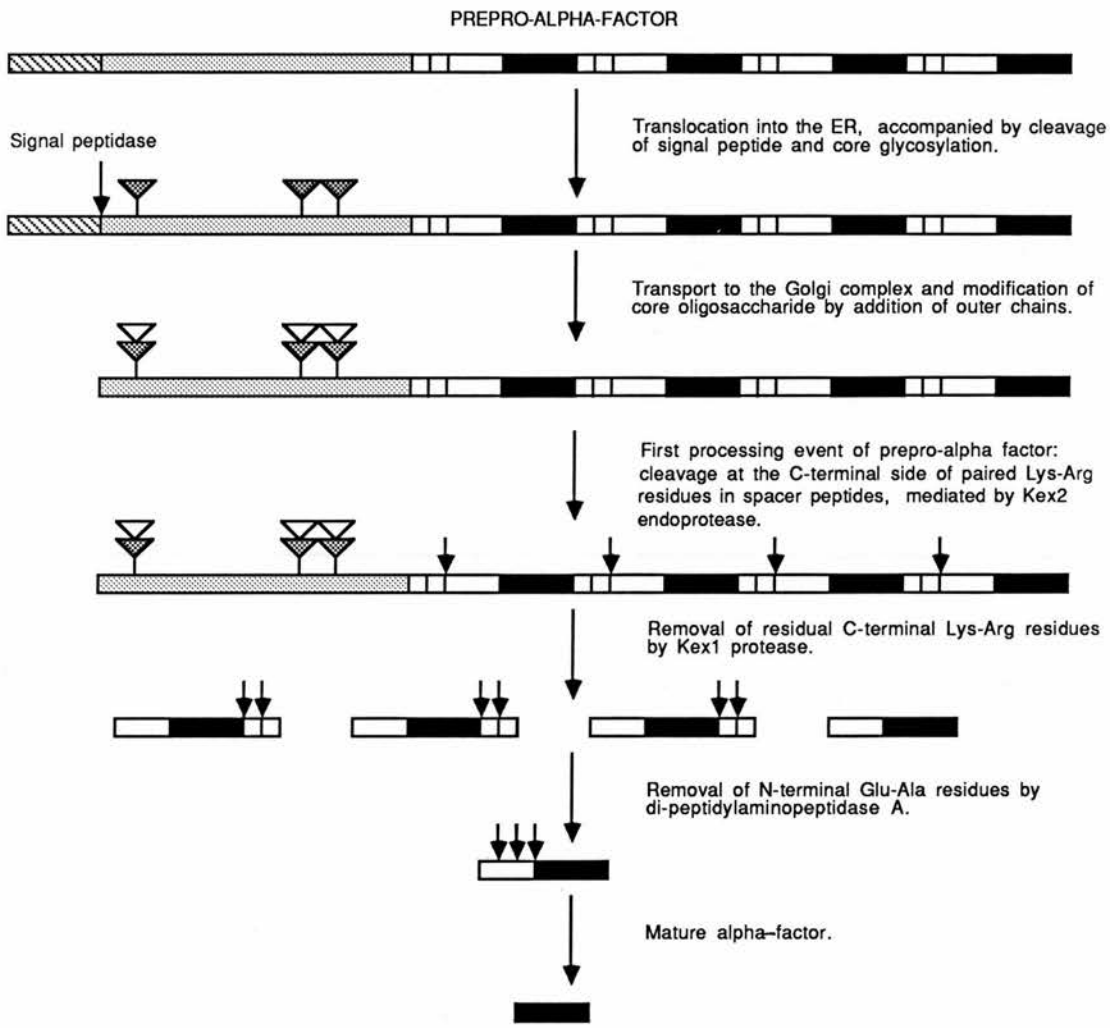
S. cerevisiae can exist as three cell types: haploid cells are either **a** or α, which can mate together to form **a**/α diploids. Both haploid cell types produce mating pheromones, **a**-factor and α–factor, which interact with cells of the opposite mating type and prepare them for mating (for review see Herskowitz 1986).

The mating pheromone α-factor is a tridecapeptide that is encoded by two genes: *MFα1* and *MFα2*, both of which encode multiple copies of the α-factor peptide, *MFα1* has four copies but *MFα2* only two, which are initially synthesised as large precursor polypeptides. Mature α-factor is generated by proteolytic processing of these polypeptides as they traverse the secretory pathway. *MFα1* encodes a polypeptide precursor of 165 amino acids (prepro-α-factor), of molecular weight 18 580, which has the following features: a pre-region which comprises a 20 amino acid signal sequence; a 60 amino acid pro-region which contains three consensus sites for the additon of N-linked oligosaccharide; and a carboxy-terminal region which encodes four copies of the mature α-factor peptide. The four repeat sequences are separated by the spacer sequence LysArgGluAlaGluAla (See fig. 1.4, Kurjan and Herskowitz 1982).

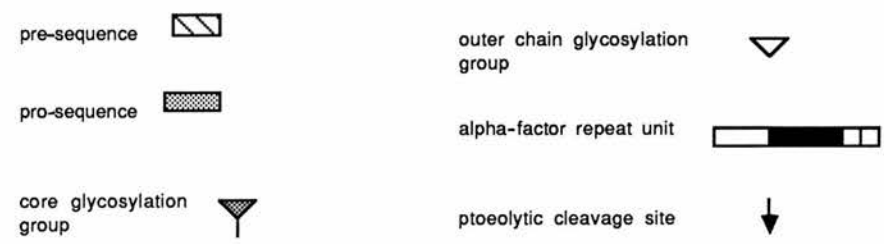
The biogenesis of this precursor, as shown in fig.1.4, was in part determined by analysis of the protein in *sec* mutants (Julius *et al.*, 1984a). The polypeptide is directed into the secretory pathway by the amino-terminal signal sequence, which is then removed by signal peptidase (Waters *et al.*, 1988). The species of pro-α-factor accumulated in a *sec18-1* mutant (blocked in ER to Golgi transport) has a molecular weight of approximately 25K. This increase in molecular weight is consistent with the addition of three core oligosaccharides to the polypeptide, at consensus sites in the pro-region. Analysis of α factor accumulated in a *sec59-1* mutant (a class B *sec* mutation) under restrictive conditions supports this: the

Fig 1.4 Biosynthesis of the mating pheromone α-factor.

This diagram shows post-translational processing of the *MFα1* gene product - prepro-α-factor. This pathway has been elucidated by biochemical and immunocytochemical identification of processing intermediates and by analysis of individual processing enzymes and their genes. See text for further details.


PREPRO-ALPHA-FACTOR
Signal peptidase
Translocation into the ER, accompanied by cleavage of signal peptide and core glycosylation.
Transport to the Golgi complex and modification of core oligosaccharide by addition of outer chains.
First processing event of prepro-alpha factor: cleavage at the C-terminal side of paired Lys-Arg residues in spacer peptides, mediated by Kex2 endoprotease.
Removal of residual C-terminal Lys-Arg residues by Kex1 protease.
Removal of N-terminal Glu-Ala residues by di-peptidylaminopeptidase A.
Mature alpha-factor.

KEY:


pre-sequence
pro-sequence
core glycosylation group
outer chain glycosylation group
alpha-factor repeat unit
ptoeolytic cleavage site

sec59-1 mutation prevents proteins from crossing the ER membrane, instead nascent polypeptides become lodged in the membrane during translocation. Regions of the proteins however, may protrude into the lumen of the ER and be used as substrates for glycosylation. Four distinct species of pro-α-factor accumulate in *sec59-1* mutants at 37°C which only differ in the number of core oligosaccharides that have been added to the polypeptide; these are thought to represent pro-α-factor with either zero, one, two, or three core oligosaccharides.

Proteolytic processing of pro-α-factor is thought to occur during transit through the Golgi, since no processing was detected in material accumulated by a *sec18-1* mutant. The first cleavage of pro-α-factor is thought to be carried out by the Kex2 endoprotease, at the pairs of basic residues in the spacer peptides, to release the four pheromone repeat units (see fig.1.4). The residual GluAla residues at the amino-terminus of each peptide are removed by dipeptidylaminopeptidase-A, and the carboxy-terminus LysArg residues by Kex1 exoprotease. This series of events must be initiated after pro-α-factor has traversed the position at which *SEC7* exerts its effect, since material accumulated in a *sec7-1* mutant at 37°C showed little evidence of processing; and by similar argument must be completed by the time α-factor is secreted since mature α-factor accumulates in a *sec1-1* mutant under restrictive conditions.

The three enzymes that are responsible for processing of pro-α-factor, ie. Kex2 endoprotease and, Kex1 and DPAP-A exoproteases have been identified, biochemically characterised, and the structural gene for each enzyme isolated (see Bussey 1988 for review). A summary of part of this data is presented in table 1.3 along with key references to which the reader is referred to for more information. This type of proteolytic maturation of proteins is a general occurence in eukaryotic cells (for review see Thomas *et al.*, 1988), but the biosynthesis of α-factor provides the first example of a eukaryotic protein in which all the biogenic precursors and the enzymes responsible for processing have been identified.

Table 1.3 Proteases that participate in the maturation of the mating pheromone α-factor

PROTEASE	ACTIVITY	GENE	COMMENTS	REFERENCES
Kex2 endoprotease	Cleavage at the C-terminal side of paired basic residues (Lys-Arg).	*KEX2*	The *KEX2* gene has been cloned by complementation of the *kex2* mutation. The DNA sequence of the gene predicts a polypeptide of 814 residues of approx mol. wt. 90K. Hydrophobic residues are are found at the N-terminus and between resdiues 675-700, which could function as a signal sequence and transmembrane domain respectively. The amino terminal 675 residues of Kex2 protease contains sequences that show homology to the subtilisin family of bacterial serine proteases, are sites for N-linked and O-linked glycosylation, and that are required for activation by Ca^{2+}. The enzyme is thought to be located in the Golgi.	Julius *et al.*, 1984a Fuller *et al.*, 1988
Kex1 exoprotease	Removal of Lys-Arg residues from C-terminus of peptides released by Kex2 cleavage	*KEX1*	A membrane associated carboxypeptidase B like activity that cleaves pairs of Lys-Arg residues from synthetic prptides has been reported in yeast. The *KEX1* gene is thought to encode this protein since the activity is absent in *kex1* mutants.The *KEX*1 gene has been cloned by complementation of the *kex1* mutation. The DNA sequence of the gene predicts a 729 residue protein of mol. wt. 82K, which shows strong homology to the serine protease CPY, infact Kex1 protease has a sequence identical to the CPY active site (GDSYAG). The enzyme is thought to be located in the Golgi.	Aschetter and Wolf 1987 Dmochowska *et al.*, 1987
Dipeptidyl amino-peptidase-A (DPAP-A)	Removal of Glu-Ala residues from N-terminus of peptides released by Kex2 cleavage	*STE13*	*ste13* mutants secrete a mixture of non-functional, incompleltely processed forms of α factor, all of which possess residual Glu-Ala residues from the spacer peptide at their amino terminus; *ste13* mutants are therefore incapable of mating (sterile). The *STE13* gene has been cloned by complementation of the *ste13* mutation. DNA sequencing predicts a polypeptide of 931 resdiues of approx 90K. Cell fractionation studies have shown that DPAPase-A is located in seperate compartment of the secretory pathway to Kex2 protease, but it is not known whether this compartment is another cisterna of the Golgi or secretory vesicles.	Julius *et al.*, 1983 Fuller *et al.*, 1988

Biogenesis of the vacuolar hydrolase- carboxypeptidase Y.

The yeast vacuole contains a number of hydrolytic enzymes, and for this reason is thought to be equivalent to the lysosome of mammalian cells (for review see Jones 1984). One vacuolar enzyme, the serine protease carboxypeptidase Y (CPY) has been studied in detail. The gene encoding CPY, *PRC1*, has been isolated and sequenced (Stevens *et al.*, 1986, Valls *et al.*, 1987). An open reading frame has been identified that could encode a protein of 532 residues, with a molecular weight of 58K. By reference to the known amino acid sequence the amino-terminus of mature CPY begins at residue 112, indicating that CPY is synthesised as an immature precursor (proCPY) containing a 111 residue pro-sequence (Martin *et al.*, 1982, Svendson *et al.*, 1982, Breddam and Svendson 1984). Residues 3-18 of this propeptide are hydrophobic and are thus thought to act as a signal sequence to direct the protein into the secretory pathway. Maturation of the protein therefore, involves two proteolytic cleavages: removal of the amino-terminal signal peptide by signal peptidase, followed by removal of the pro-sequences to release active CPY. Conversion of proCPY to CPY occurs in the vacuole and is dependent upon the *PEP4* gene product (proteinase A, Ammerer *et al.*, 1986, Woolford *et al.*, 1986): in *pep4* mutants CPY appears as an inactive protein of molecular weight 69K, of which 10K is N-linked carbohydrate; whereas in wild type cells the pro-sequence is removed to produce a protein of molecular weight 61K, of which 10K is also accountable to carbohydrate (Hasilik and Tanner 1978).

In similar fashion to invertase, CPY also moves rapidly from its site of synthesis to the vacuole, taking approximately 6 minutes, and thus detailed information about its biogenesis has only been gained by analysis in *sec* mutants (Stevens *et al.*, 1982). Those mutants that accumulate ER at 37^{o}C (e.g *sec18-1*) also accumulate an immature form of CPY (p1CPY) of molecular weight 67K, of which 8K is N-linked oligosaccharide, consistent with the addition of core oligosaccharide units at four positions in the mature protein. From the molecular weight of p1CPY, it is not

possible to resolve whether the amino-terminal signal peptide is cleaved in the ER, although recent evidence supports this (Blachly-Dyson and Stevens 1987).

The two mutants that accumulate Golgi at 37°C (*sec7* and *sec14*), exhibit different responses to restrictive conditions: *sec7-1* mutant cells accumulate p1CPY, identical to that seen in a *sec18-1* mutant; whereas a *sec14-3* mutant accumulates two different proteins, p1CPY and a second protein of molecular weight 69K (p2CPY) in equal amounts. The difference in molecular weight of p1CPY and p2CPY is attributable to addition of outer chains to N-linked oligosaccharide during transit through the Golgi. This clearly suggests that *SEC7* and *SEC14* gene products exert their effects at different positions in the Golgi complex. Those *sec* mutants that accumulate secretory vesicles (e.g *sec1-1*) block the pathway sometime after proteins have traversed the Golgi, and do not prevent maturation of CPY into the vacuole. In yeast therefore, a direct pathway exists from the Golgi to the vacuole, similiar to that one described from the Golgi to the lysosome in mammalian cells.

Purification of secretory organelles from *sec* mutants.

The low abundance of secretory organelles makes their purification from wild type cells problematical; the *sec* mutants however, provide an enriched supply of these organelles thereby facilitating such experiments. Two groups have reported purification of secretory vesicles from late blocking *sec* mutants at the non-permissive temperature: Walworth and Novick (1987) utilised a *sec6-4* mutant and Holcomb *et al.,* (1988) a *sec1-1* mutant. Examination of purified vesicles by electron microscopy revealed that purified vesicles were not coated, either by clathrin or some other protein which is entirely consistent with the observation that secretion of invertase is not perturbed in *chc1* mutants (see section 1.1.8, Payne and Schekman 1985).

Further analysis also demonstrated that plasma membrane proteins, e.g the plasma membrane Na^+/K^+ ATPase, and soluble proteins, e.g acid phosphatase, are transported to the cell surface in the same vesicles (Holcomb *et al.,* 1988, Brada and Schekman 1988); and also that vesicles accumulated in the two different *sec* mutants have similiar polypeptide compositions. Walworth and Novick (1987) analysed purified vesicles by SDS-PAGE, and observed three predominant protein bands of approximately molecular weight 110K, 40-45K, and 18K when proteins were visualised with Coomassie blue. The largest protein was specifically labelled with [^{3}H] mannose, and found to partition into the aqueous phase of Triton X114, strongly suggesting it to be a soluble glycoprotein that forms part of the vesicle cargo. The proteins of molecular weight 40-45K and 18K however, partition into the detergent phase of Triton X114, and have cytoplasmic domains that are suseptible to proteases. This is consistent with them being transmembrane proteins, but the possibility remains that they are attached to the periphery of the vesicle in the same fashion as Sec4p. Analysis of *sec1-1* accumulated vesicles by SDS-PAGE resulted in a very similiar polypeptide profile, raising the possibity that this pattern is constant in the vesicles accumulated in any late *sec* mutant.

Many of the *sec* mutants are also defective in endocytosis.

The biochemical evidence reviewed in section 1.2, supports the hypothesis that the endocytic and secretory pathways pass through common organelles and are therefore connected. In the text below I describe further genetic evidence, which implies that several gene products that perform an essential role in secretion are also required for endocytosis.

Riezman (1985) reported that *S. cerevisiae* has a functional endocytic pathway. He used a synthetic dye, lucifer yellow carbohydrazide, that had previously been exploited as a marker of fluid phase endocytosis in mammalian cells, and demonstrated that it was internalised by actively growing yeast cells. The

characteristics of internalisation are indicative of endocytic uptake, as opposed to diffusion or directed transport across the plasma membrane: the accumulation of lucifer yellow is temperature and energy dependent, and the amount of material internalised is directly proportional to the external concentration of lucifer yellow (ie it is not saturable). Using this as an assay the ability of the *sec* mutants to endocytose lucifer yellow was determined (Riezman 1985). All 23 *sec* mutants internalised lucifer yellow at the permissive temperature, although several accumulate less than wild type. At 37°C however, many of the mutants are deficient in endocytosis: the ten mutants that accumulate SV's are all defective for accumulation of lucifer yellow in the vacuole; of the two mutants that accumulate Golgi, *sec7* is defective but *sec14* is not; and of the nine mutants that accumulate ER, *sec18* and *sec23* are also defective in endocytosis.

This is a clear implication of a broad overlap of functions required for movement of membrane during both endocytosis and secretion, although the two processes are seperate since mutants (*end*) that are defective in endocytosis but not secretion have been isolated (Chvatchko *et al.*, 1986). This could either be due to a simple requirement for *SEC* gene products in endocytosis and secretion, or a more complicated relationship where part of the endocytic pathway is obligatorily coupled to certain stages in the secretory pathway.

1.3.3. Résumé

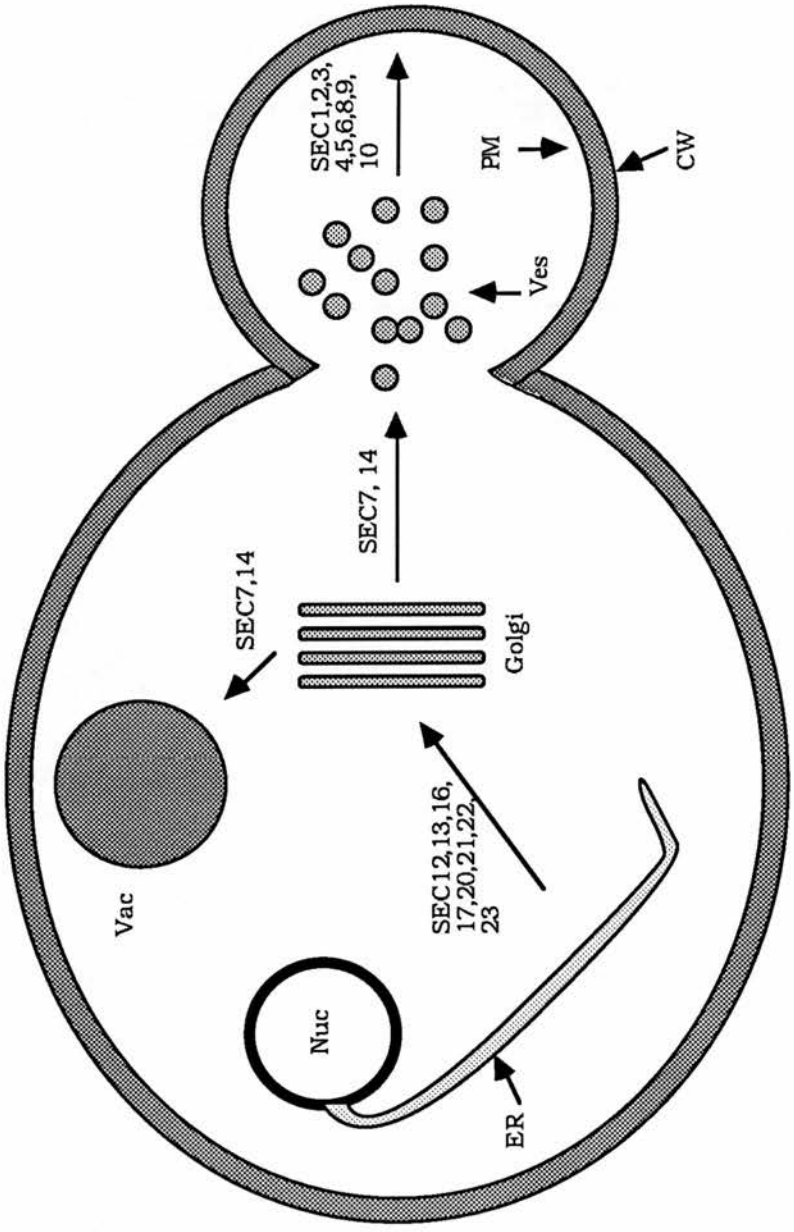
The genetic and biochemical studies described above have led to the proposal of a model of secretion that is fundamentally no different to that for the secretion of proteins from mammalian cells (Schekman 1985, fig 1.5), in that it is a sequential movement from the RER through the Golgi, to secretory vesicles which release their contents by exocytosis. Entry into the secretory pathway is envisaged to occur by cotranslational translocation, similiar to that previously described. Translocation of proteins across the ER membrane in yeast is dependent upon an amino terminal

signal peptide, although as yet no reports have appeared in the literature describing SRP or SRP receptor. Following entry into the ER, proteins are transported to the Golgi complex, via hypothetical vesicles.

The Golgi complex represents a branch point within the pathway: vacuolar proenzymes are segregated and transported to the vacuole, whilst secreted and exported proteins are packaged into vesicles for transport to the plasma membrane. The yeast Golgi therefore seems to share many of the responsibilities of the mammalian Golgi. Despite this central role however, the organelle remains relatively "unexplored" in yeast: as yet there is no evidence for compartmentalisation of the organelle into *cis*, medial, and *trans* cisternae, or some other alternative morphology. Ultimately however, yeast must use mechanisms similiar to those of mammalian cells to transport proteins through the Golgi, since a yeast cytosolic fraction can substitute for its mammalian counterpart in the *in vitro* system of Dunphy and colleagues (1986). Exit from the Golgi complex occurs by a budding mechanism to yield secretory vesicles, which fuse with the plasma membrane to release their contents. Histochemical staining of newly synthesised acid phosphatase and invertase however, reveals that in actively growing yeast cells secretion is polarised to the region of cell growth: exocytotic events are not randomly distributed over the cell surface, but are localised to the developing bud (Tkacz and Lampen 1972, Linnemans *et al.*, 1977, Field and Schekman 1980).

Fig 1.5 The yeast secretory pathway.

Proteins that are secreted by *S. cerevisiae* are thought to traverse a pathway similiar to that described for mammalian systems. Proteins enter the pathway by co-translational translocation across the membrane of the ER, after which they are carried from the ER to the Golgi and then to the plasma membrane; all transport is thought to be mediated by vesicular translocation. Temperature sensitive mutations (*sec*)have been isolated that block this pathway at a precise location, reflected by an accumulation of an organelle of the secretory pathway under restrictive conditions. The position at which the corresponding wild type gene products are thought to function is shown. See text for further details. CW (cell wall), ER (endoplasmic reticulum), Nuc (nucleus), PM (plasma membrane), Vac (vacuole), Ves (post-Golgi secretory vesicles).


SEC1,2,3,
4,5,6,8,9,
10
PM
CW
Ves
SEC7, 14
SEC7,14
Golgi
Vac
SEC12,13,16,
17,20,21,22,
23
Nuc
ER

1.4 INVESTIGATIONS OF *SEC* ENCODED GENE PRODUCTS AND OTHER PROTEINS THOUGHT TO BE INVOLVED IN SECRETION.

The isolation of the *sec* mutants has provided a foothold in the study of secretion in yeast. Characterisation of mutant phenotypes, isolation of the corresponding wild type gene, identification of suppressor mutations, and the development of cell free systems will allow the functions that the *SEC* encoded proteins fulfill to be elucidated. Several groups have decided to follow this pathway of investigation, and have successfully isolated and characterised a number of *SEC* genes by complementation of the corresponding *sec* mutant. These results are presented in table 1.4.

1.4.1 The *SEC4* gene encodes a GTP binding protein that is found in association with secretory vesicles and the plasma membrane.

Analysis of the *SEC4* gene deserves special consideration since it has provided new clues into some of the fundamental mechanisms that may be employed during secretion. The *SEC4* gene was originally isolated in an attempt to clone the *SEC15* gene: a genomic library formed in the plasmid YCp50 (maintained at single copy, Rose *et al.*, 1987) was used to transform a *sec15-1* strain, with selection for temperature resistant strains. Subsequent genetic analysis however, revealed that the isolated fragment of genomic DNA did not correspond to the *SEC15* locus. This is most easily explained by an unlinked gene being isolated, whose product fulfills a function similiar to the *SEC15* gene product, which when duplicated can suppress the *sec15-1* mutation. Further analysis revealed that the DNA fragment carried the *SEC4* gene.

During these studies some level of genetic interaction was also demonstrated between the *SEC4* gene and many other *sec* mutants (Salminen and Novick 1987). Representatives of all the *sec* mutants were transformed with a YCp50 based

Table 1.4 Summary of findings from analysis of certain *SEC* genes.

GENE	ORGANELLE ACCUMULATED BY MUTANT	CHARACTERISITCS	REFERENCE
SEC4	Post-Golgi secretory vesicles.	*SEC4* encodes a GTP binding protein of mol wt 23K. Sec4p does not have an N-terminal signal sequence, or other internal hydrophobic domains, but is found in strong association with secretory vesicles and the plasma membrane. See text for further details. Function unknown.	Salminen and Novick 1987. Goud *et al* 1988
SEC7	Golgi like structure (Berkeley Bodies)	An ORF of 2008 residues has been identified by DNA sequencing which could encode a protein of mol wt 228K. Immunoprecipitation of Sec7p identifies a polypeptide of this size, which is phosphorylated and distributed in both particulate and soluble fractions of the cell. Function unknown.	R. Schekman personal communication
SEC11	None	At 37^{o}C *sec11* cells accumulate core glycosylated material in the lumen of the ER; this material has not been processed by signal peptidase. The *SEC11* gene encodes a protein of 167 residues, of mol wt 18 825, that is predicted to be an integral membrane protein. It is proposed that Sec11p is a component of signal peptidase.	Bohni *et al* 1988
SEC15	Post-Golgi secretory vesicles	DNA sequencing has identifed an ORF sufficient in size to encode a protein of approx mol wt 100K, but immunoprecip-itation of Sec15p suggests a mol wt of 115K; the reason for this discrepency is not understood. Sec15p does not have any hydrophobic sequences to direct it into a membrane, but the protein is found associated with the particulate fraction of the cell. Immunofluorescence expts show Sec15p distributed over the peripheryof the cell in a punctate pattern. Sec15p may be associated with the actin cytoskeleton. Function unknown.	A. Salminen personal communication

GENE	ORGANELLE ACCUMULATED BY MUTANT	CHARACTERISITCS	REFERENCE
SEC12	Endoplasmic Reticulum	The *SEC12* gene encodes a polypeptide of 471 residues, of approx mol wt 70K. Sec12p is an integral membrane protein that undergoes N-linked, and probably O-linked glycosylation. Sub-cellular fractionation and immunoelectron-microscopy have localised Sec12p to ER and Golgi membranes. Function unknown.	R. Schekman personal communication
SEC18	Endoplasmic Reticulum	DNA sequencing has revealed that the *SEC18* gene encodes an polypeptide of mol wt 83.9K; but *in vitro* transcription and translation of *SEC18* leads to the synthesis of two polypeptides, of mol wt 84K and 82K. These two species are thought to be the product of translation initiation at different in-frame ATG codons. Neither polypeptide has a N-terminal signal sequence, yet some Sec18p is found associated with membrane. Function unknown.	S. Emr personal communication
SEC23	Endoplasmic Reticulum	The *SEC23* gene encodes a protein of approx mol wt 84K, that is found attached to the periphery of ER and Golgi. Function unknown.	R. Schekman personal communication

Numerous *SEC* genes have been isolated by complementation of the corresponding *sec* mutation, and subsequently analysed; this table summarises some of the results. All the *SEC* genes studied so far have been found to encode proteins whose function is absolutely requied for cell viability. Further details of the *SEC4* and *SEC15* gene products are given in the text.

plasmid, carrying the *SEC4* gene, and tested for the ability to grow at 25°C, 33.5°C, and 37°C. At 37°C duplication of the *SEC4* gene was found to suppress the *sec2-41* mutation, in addition to *sec4-8* and *sec15-1* mutations. When growth was tested at the restrictive temperature of 33.5°C, the *sec1-1*, *sec5-24*, *sec8-9*, *sec10-2*, and *sec19-1* mutations were also suppressed to some extent. With the exception of *sec19-1*, all of these mutations block fusion of secretory vesicles with plasma membrane, and thus fall into the same class as *sec4-8*. As yet it is unknown where *sec19-1* exerts its effects since *sec19* mutants accumulate ER, Golgi, and SV's under restrictive conditions (Novick *et al.*, 1980). It has also been demonstrated that combination of the *sec4-8* mutation with any of the above mutations, with the exception of *sec1-1*, is lethal at the permissive temperature, providing further evidence of strong genetic interaction between these components (Salminen and Novick 1987).

The nucleotide sequence of the *SEC4* gene has been determined (Salminen and Novick 1987), and predicts a protein of 215 amino acids (Sec4p), of molecular weight 23,479, which has significant similarity to all *ras* transforming proteins. Similarity was strongest in the regions of the *ras* proteins that have been implicated in GTP binding and hydrolysis, and indeed Sec4p possesses the three sequence motifs that have been correlated with GTP binding function (McCormick *et al.*, 1985, Jurnak 1985). Using a TrpE-Sec4p fusion protein as an immunogen, antibodies have been raised that specifically recognised a protein of molecular weight 23.5K in a crude mixture of yeast proteins (Goud *et al.*, 1988). It was possible to radiolabel this protein with [^{32}P]-GTP confirming its ability to bind GTP. Subcellular fractionation experiments revealed that Sec4p exists in both the soluble and particulate fractions of the cell - approximately 85% Sec4p sedimented with membranes. In wild type cells 10-20% of the membrane bound Sec4p was found associated with secretory vesicles, whilst the remaining Sec4p purified with plasma membrane; when this distribution was analysed in *sec6-4* (SV) mutant cells at 37°C the amount of Sec4p associated with secretory vesicles increased to 50%. Goud

et al., (1988) propose that Sec4p initially associates with secretory vesicles and is then transferred to plasma membrane as a result of exocytotic events.

Membrane associated Sec4p exhibits characteristics typical of integral membrane proteins: Sec4p can not be washed off membranes by exposure to high pH, and in partitioning experiments using the detergent Triton X114 the protein partitions into the detergent phase (Bordier 1982, Goud *et al.,* 1988). This is surprising since the polypeptide does not possess an amino-terminal signal peptide, or internal hydrophobic domains, that could direct translocation into a membrane. The protein must therefore undergo some post translational modification or conformational change in order to become attached to a membrane. In mammalian systems it is thought that fatty acid acylation may mediate attachment of some proteins to membrane (Magee and Schlessinger 1982), e.g for *ras* proteins, a palmitate moiety is added in a thioester linkage to a cysteine located in the sequence Cys-A-A-X, where A is any aliphatic amino acid and X the carboxy-terminus (Sefton *et al.,* 1982, Buss and Sefton 1986); Sec4p does not share this sequence but it does have the unusual C-terminal sequence of SerAsnCysCys. Another yeast protein that may be involved in secretion, Ypt1p (see below) also has two cysteine residues at its carboxy-terminus (GlyGlyCysCys); in this case the two cysteine residues are essential for function, and mediate palmityolation of the protein, allowing its association with membrane (Molenaar *et al.,* 1988). It is tempting to speculate that Sec4p is attached to membrane in similiar fashion, although attempts to radiolabel Sec4p with [^{3}H] palmitate however, have proven unsuccessful (Goud *et al.,* 1988).

1.4.2 The *YPT1* gene is a *ras* homologue and encodes a protein that may be invovled in secretion.

The protein that Sec4p resembles most closely is another yeast *ras* homologue - Ypt1p, product of the *YPT1* gene. Ypt1p is 206 amino acids in length and has 48% identity to Sec4p. This high level of similarity is maintained throughout the protein, including those regions responsible for binding of GTP. This homology is made even more intriguing by recent reports that Ypt1p may also perform an important function during secretion. The *YPT1* gene was originally identified as an open reading frame located between the structural genes encoding actin (*ACT1*) and tubulin (*TUB2*) on chromosome VI (Gallwitz *et al.,* 1983). The ORF is 618bp long, sufficient to encode a polypeptide of 206 amino acids with a molecular weight of 23 213. As yet no function can be assigned to this protein, but as with Sec4p, Ypt1p bears significant resemblance to mammalian and viral *ras* proteins: over a region of 168 residues Ypt1p shows 38% homology with the 189 residues of the human *c-ras* gene products, suggesting that these proteins have similiar secondary and tertiary structure, and perhaps function (Gallwitz *et al.,* 1983). Consistent with Ypt1p being a *ras* protein the protein can bind and hydrolyse GTP (Wagner *et al.*, 1987).

In an attempt to elucidate the function of Ypt1p, two groups have undertaken genetic analyses of the *YPT1* gene (Schmitt *et al.,* 1986, Segev and Botstein 1987). Both groups confirm that the*YPT1* gene is essential for cell viability since a *ypt1* null mutation is lethal. To further investigate the function of Ypt1p, both groups chose to examine the effects of depriving growing cells of functional Ypt1p. To achieve this Schmitt *et al.,* (1986) replaced the chromosomal *YPT1* gene with a *YPT1* gene that had been placed under the control of the *GAL10* promoter, allowing high levels of *YPT1* expression by growth of yeast cells in media containing galactose as the sole carbon source; or expression can be completely repressed by using glucose as a carbon source. Alternatively, Segev and Botstein (1987) used site directed mutagenesis to construct a cold sensitive mutant allele of *YPT1*, which was

transplaced onto the chromosome in place of the wild type *YPT1* gene. The *ypt1-1* mutation is recessive and is lethal at the restrictive temperature of 14°C.

When exponentially growing cells were deprived of fuctional Ypt1p, either by repression of the *GAL10* promoter or incubation of the *ypt1-1* mutant at 14°C, many phenotypic changes occurred. Cell growth ceased after several hours in the absence of Ypt1p; after 24hrs, examination of arrested cells revealed that they were significantly larger than wild type cells, but in contrast to *cdc* mutants they had not arrested with a unique morphology. However, Segev and Botstein (1987) observed that arrested cells had replicated their DNA and thus contained two haploid units of DNA per cell. They postulated therefore that Ypt1p is required for cell division, specifically after most of the nuclear DNA has been replicated, and proceded to examine the effects of Ypt1p deprivation in synchronous cultures. Unbudded *ypt1-1* cells were collected from Ficoll gradients and then incubated at the restrictive temperature (14°C) for several hours. Examination of arrested cells then revealed that the majority had arrested with a unique morpholgy (mother cells with small buds), consistent with Ypt1p being involved in the cell cycle.

Recently, Segev *et al.,* (1988) have published results that link Ypt1p with the secretory apparatus. Electron microscopy of the *ypt1-1* mutant, revealed that at the restrictive temperature (14°C), and to a lesser extent at the permissive temperature (30°C), mutant cells experience an intracellular accumulation of aberrant membranes. Two principal types of membrane were observed: long stacks of cylindrical membranes that could represent abnormal ER, and large vesicles that resembled the Berkeley bodies accumulated by *sec7* and *sec14* mutants. Consistent with these findings, secretion of invertase from *ypt1-1* cells is inhibited at 14°C, resulting in an intracellular accumulation of enzyme, that was only partially glycosylated. Invertase accumulated by *ypt1-1* cells at 14°C migrates more slowly by SDS-PAGE than invertase accumulated by a *sec18-1* mutant at 37°C, but faster than invertase from wild type cells, suggesting that the *ypt1-1* mutant is defective

either in the transfer of material from the ER to Golgi, or within the Golgi complex.

The subcellular location of Ypt1p has been visualised by immunofluorescence using anti-Ypt1p antibodies, and provides tantalising evidence that the protein is localised to the Golgi complex. Wild type cells display a punctate pattern within the cytoplasm, with intense staining also in the region of the bud. To test that this pattern represents the distribution of a secretory organelle, immunofluorescence was repeated in numerous *sec* mutants: the same punctate pattern was seen in *sec18-1* and *sec1-1* mutant cells at both the permissive and restrictive temperatures, but only at the permissive temperature in *sec7-1* mutants. At the restrictive temperature *sec7-1* mutants displayed aggregated structures that differed significantly from wild type cells, consistent with Ypt1p being localised to the Golgi complex (Segev *et al.*, 1988).

1.4.3. A putative role for the cytoskeleton in secretion.

In *S. cerevisiae*, the first implication that the cytoskeleton was involved in secretion came from investigations of actin. Actin is encoded by a single gene (*ACT1*) that has been cloned, sequenced, and is essential for cell viability (Gallwitz and Seidel 1980, Gallwitz and Sures 1980, Ng and Abelson 1980, Shortle *et al.*, 1982). To study the effects of actin deprivation on yeast cells three temperature sensitive mutations, *act1-1*, *act1-2*, and *act1-3* were constructed *in vitro* and then used to replace the wild type *ACT1* chromosomal gene (Shortle *et al.*, 1984). Analysis of the phenotype displayed by *act1-1* and *act1-2* mutant strains under restrictive conditions revealed a defect in secretion: after thirty minutes at 37°C approximately 80% of invertase was intracellular; this material was fully glycosylated consistent with a late block of the secretory pathway. Examination of mutant strains by electron microscopy also demonstrated an intracellular accumulation of post-Golgi secretory vesicles similar to those seen in some of the *sec* mutants (Novick and Botstein 1985). The actin cytoskeleton could therefore mediate transport of post-Golgi

secretory vesicles to the plasma membrane.

The *YPT1* gene product may also exert its effect through the cytoskeleton. The fact that the *YPT1* and *TUB2* genes are adjacent has led to speculation that Ypt1p is a GTP binding protein that interacts with tubulin during microtubule formation, which is GTP dependent. In support of this hypothesis, cells that have been deprived of Ypt1p exhibit abnormal microtubule structure and distribution (Scmitt *et al.,* 1986, Segev and Botstein 1987). Microtubules have been visualised in wild type cells by immunofluorescence and are seen to originate from two spindle pole bodies and extend into the nucleus, where they either attach to the chromosomes or run from pole to pole to form the spindle (Kilmartin and Adams 1984). In addition, other microtubules extend into the cytoplasm and the developing bud, whose function is thought to ensure correct orientation of the spindle before its elongation and chromosome separation. Cells deprived of Ypt1p however, show an abnormal lengthening of cytoplasmic microtubules, accompanied by alterations of the spindle which also appears to be of uneven thickness. Prolonged deprivation leads to large scale breakdown of the mitotic spindle, and ultimate dispersion of nuclear material throughout the cytoplasm. In many of these cells, several "rod"-like microtubules, microtubules forming extensive loops, and amorphous pathches of aggregated tubulin can also be seen.

The *ypt1-1* mutation also has an effect on actin morphology (Segev and Botstein 1987): in unbudded wild type cells, actin appears as numerous dots or patches distributed over the cell surface when visualised by immunofluorescence, whilst budded cells are stained asymmetrically, ie extensive staining of the bud with faint cables passing into the mother cell (Adams and Pringle 1984). The distribution of actin in the bud changes as the bud matures: in very small buds actin is usually localised to a cluster of individual spots, which increase in number as the bud grows; but in fully developed buds actin becomes localised to the neck region. In *ypt1-1* mutants that had been incubated at 14^{o}C for 24hrs there was no evidence of

asymmetrical staining in budded cells, instead actin spots were seen with equal frequency in both the bud and mother cell.

Schmitt *et al.,* (1988) argue that the pleiotropic effects resulting from Ypt1p deprivation (ie: inhibition of cell division, disorganisation of microtubules and actin deposition, and accumulation of secretory organelles) is most probably due to a defect in a protein that regulates a cellular component required for all these processes. They postulate that this component is calcium because in certain eukaryotic cells secretion is dependent on calcium (Burgoyne 1987, Howell *et al.,* 1987), and calcium is probably involved in regulating microtubule assembly (Schliwa *et al.,* 1981, Keith *et al.,* 1983). In yeast, a calcium binding protein is required for spindle pole body duplication (Baum *et al.,* 1986), calcium controls actin assembly (Greer and Schekman 1982), and calcium promotes the association of secretory vesicles with plasma membrane (Henschke *et al.,* 1983). In support of this hypothesis mutant strains carrying a temperature sensitive *ypt1*^{ts} allele, can be rescued by increasing the extracellular levels of calcium, suggesting that Ypt1p may participate in modulating intracellular levels of calcium; but it is unclear how this could be brought about since the major reservoir of calcium is the vacuole (Oshumi and Anraku 1983, Eilam *et al.,* 1985), yet the protein is localised to the Golgi (Segev *et al.,* 1988).

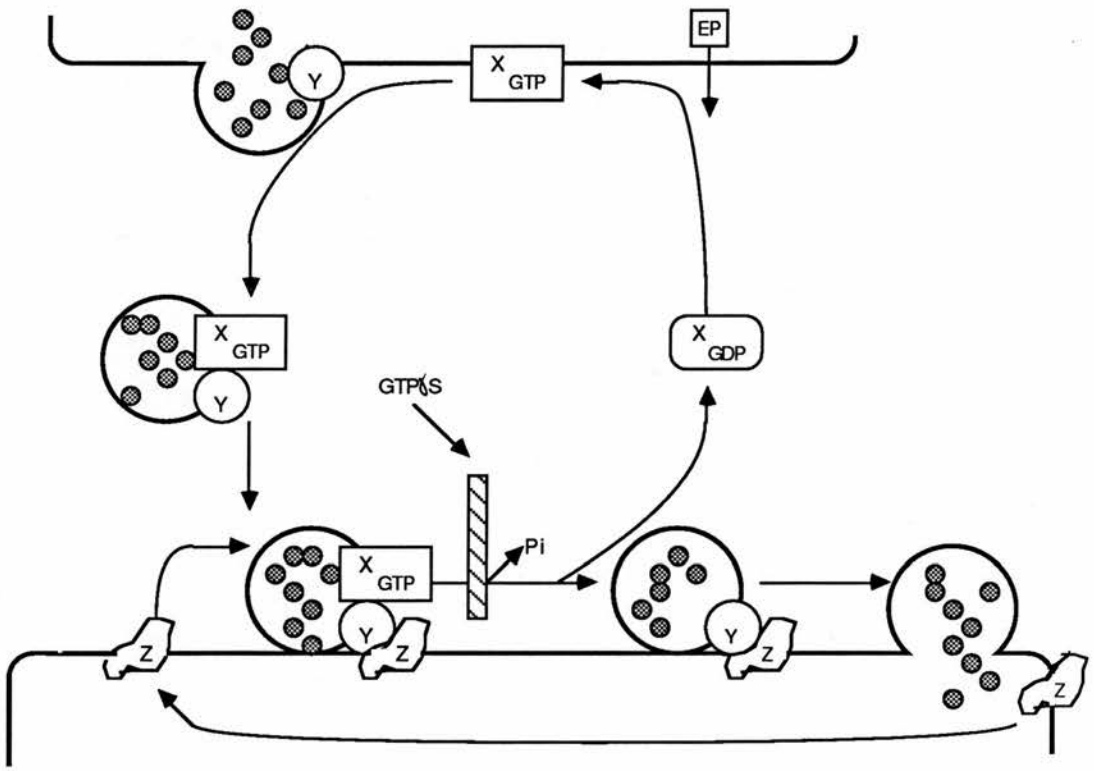
1.4.4 GTP binding proteins also function during protein transport in mammalian cells.

The discovery that, in yeast, GTP binding proteins may perform an essential function during secretion is an exciting new insight. Previously, only regulatory functions have been ascribed to guanine-nucleotide binding proteins, such as signal transduction in receptor systems and in growth control. This new role for GTP binding proteins is probably not restricted to yeast, since proteins homologous to Ypt1p have been identified in mammalian systems (Touchot *et al.,* 1987, Haubruck

et al., 1987). The closest homologue, mouse *ypt1*, encodes a polypeptide of molecular weight 23K, which has 71% of its residues identical to yeast Ypt1p. Moreover, anti-yeast Ypt1p antibodies recognise a protein of the same approximate molecular weight in mouse fibroblasts, that appears to be localised to the Golgi complex since it fractionates with crude Golgi preparations, and also decorates an internal organelle resembling the Golgi by immunofluorescence (Segev *et al.*,1988)
To assess the importance of GTP binding proteins in protein transport through the Golgi complex, the *in vitro* transport system of Rothman and colleagues (see section 1.1.3) was subjected to GTPγS and AlF_4^- (Melancon *et al.*, 1987). Both of these compounds are known activators of mammalian G proteins (see Gilman 1987), and also GTPγS is a non hydrolysable analogue of GTP, such that GTP binding proteins remain permanently activated. Both compounds inhibited transport of VSV G glycoprotein from donor to acceptor membranes. Further analysis revealed that it is probably some function of the acceptor membranes that is inhibited, since pre treatment of only donor membranes or cytosol had marginal effect on transport. Also, EM analysis of transport reactions that had been arrested, revealed an accumulation of non-clathrin coated buds and vesicles, suggesting that transport is inhibited due to an inability of donor vesicles to fuse with acceptor membrane. The function which GTP binding proteins fulfill during protein secretion is unknown, but consistent with all the data is a model in which GTP binding proteins mediate interaction between proteins (address tags) on/in the membrane of a transport vesicles with the appropriate acceptor membrane (Bourne 1988) (see fig. 1.6 and legend for full description).

Figure 1.6 **Possible function of GTP-binding proteins in vesicular translocation.**

This model has been proposed by Bourne (1988) to explain the function of GTP-binding proteins in intracellular membrane transport. A transport GTPase (X) mediates vectoral transport of vesicles between different membrane compartments: X_{GTP} recognises a protein (Y) on/in the membrane of a budding vesicle. The $X_{GTP}Y$ complex directs interaction (via Y) with a docking protein (Z) in/on the appropriate acceptor membrane, at which point GTP hydrolysis releases X_{GDP}, leaving the vesicle-YZ complex in a position to initiate membrane fusion. X_{GDP} is then recycled by conversion to X_{GTP}, a reaction catalysed by a guanine nucleotide exchange protein (EP) located on the donor membrane. In this model Bourne proposes that the GTP binding protein (X, ie. Sec4p or Ypt1p) ensures that vesicles tagged by an address label (Y) are delivered to the correct destination, tagged by another address label. The hatched box represents the position at which events could be blocked by GTPγS


Donor Membrane
EP
Y
X GTP
X GTP
Y
X GDP
GTPγS
Pi
X GTP
Y
Z
Y
Z
Y
Z
Z
Acceptor Membrane

1.5 OUTLINE OF THE PROJECT.

Mutant strains of yeast that are temperature sensitive for protein secretion provide a foothold for investigations of the secretory pathway. These strains presumably harbour a mutation in a gene whose product performs an essential function during transport of proteins from the ER to the plasma membrane. Mutations in the *SEC1* gene result in an intracellular accumulation of post-Golgi secretory vesicles under restrictive conditions. This suggests that the *SEC1* gene product is required in the late stages of the secretory pathway where secretory vesicles fuse with the plasma membrane. The aim of this project was to isolate and characterise the *SEC1* gene and its product. The *SEC1* gene was chosen because the *sec1-1* mutant is the best characterised late-blocking *sec* mutant. It is hoped that the information gained from these experiments will provide a framework for future biochemical investigations of the events which occur during fusion of secretory vesicles with the plasma membrane.

Chapter Two.

Materials and Methods.

2.1 Materials

Chemicals.

All chemicals were obtained from BDH chemicals, Sigma Chemical Co, or Fisons Ltd, Loughborough.

Radiochemicals.

[^{35}S]-dATP was obtained from either Amersham International plc (650Ci/mmol) or New England Nuclear (500Ci/mmol). [^{35}S] methionine (1115Ci/mmol) and [^{32}P]-dATP (>3000Ci/mmol) was from Amersham International plc. All radiolabelled compounds were used as supplied by the manufacturer.

Enzymes.

All DNA modification enzymes including restriction endonucleases, phage T4 DNA ligase, Bal31 exonuclease, and the Klenow fragment of DNA polymerase I were obtained from Bethesda Research Laboratories (BRL). Zymolyase 100T was from the Seikagaku Kogyo Co. Ltd; Japan. Lysozyme was from Sigma Chemical Co.

2.2 Bacterial and yeast strains.

All the strains of *E. coli* and *S. cerevisiae* used in this work are listed in table 2.1, with their relevant properties. Haploid MEY strains of *S. cerevisae* carrying mutant *sec* alleles were constructed by crossing *sec* mutant strains, obtained from the Yeast Genetic Stock centre, with either DBY746 or DBY747.

Table 2.1 Bacterial and yeast strains.

Bacterial	Genotype	Source
E. coli NM522	Δ (*lac-proAB*), *his*Δ5, (r_k^-, m_k^+), *thi*$^-$, *supE*, F' *proAB*, *lacI*^{Q}ZΔM15.	Gough and Murray (1983)
E. coli 5K	*thi*-1, *thr*-1, *leuB*6, *lacY*1, *hsdR*, *supE*, *tonA*.	A. Boyd
E. coli 159	*gal*, *uvrA*, *rpsL*, *sup*^{o}	G. Plastow.
Yeast		
DBY746	*MAT*α; *leu2,3-112; his3-11,15; trp1-289; ura3-52.*	J. Hicks
DBY747	*MAT***a**; *leu2,3-112; his3-11,15; trp1-289; ura3-52.*	J. Hicks
ABY12	*MAT*α; *sec1-1; leu2-3,112; his3-11,15.*	A. Boyd
MEY1	*MAT***a**; *trp1-289; ura3-52.*	This study
MEY12	*MAT*α; *sec1-1; his3-11,15; leu2-3,112; ura3-52.*	
MEY121	*MAT***a**/*MAT*α; *sec1-1/SEC1; trp1-289/TRP1 leu2-3,112/LEU2; his3-11,15/HIS3; ura3-52/ura3-52.*	This study
MEY230	*MAT*α; *sec2-56*; *his3-11; leu2-3,112; trp1-289.*	This study
MEY301	*MAT***a**; *sec3-2; his3-11; leu2-3,112; ura3-52.*	This study
MEY511	*MAT*α; *sec5-24; leu2-3,112.*	This study
MEY613	*MAT*α; *sec6-4*; *leu2-3,112; ura3-52.*	This study
MEY836	*MAT***a**; *sec8-6*; *leu2-3,112; ura3-52.*	This study
MEY1007	*MAT***a**; *sec10-2*; *his3-11; leu2-3,112; trp1-289, ura3-52.*	This study
MEY1503	*MAT*α; *sec15-1, trp1-289; ura3-52.*	This study
RY26	MATα; *ura3-1,3*; *ade-1*; *ade-2*; *tyr-1*; *his7*; can^R; gal$^-$; *rna2-1.*	J. Beggs
FL100	*MAT***a**, gal$^-$	J. Beggs

2.3 Media.

In general bacterial cultures were grown in complete medium (Luria broth, LB), containing 1% (w/v) Bacto tryptone, 0.5% (w/v) Bacto yeast extract, and 0.5% (w/v) NaCl. When appropriate the following antibiotics were added: ampicillin (100μg/ml), kanamycin (80μg/ml), and tetracycline (20μg/ml). When solid medium was required Bacto agar was added to the above media at a concentration of 1.5% (w/v). Bacterial strains were routinely stored on LB agar plates at 4°C, except for *E. coli* NM522 which was grown on minimal medium to maintain selection of the F' plasmid: this medium was 0.6% (w/v) Na_2HPO_4, 0.3% (w/v) KH_2PO_4, 0.05% (w/v) NaCl, 0.1% (w/v) NH_4Cl; this solution was autoclaved and on cooling 2ml 1M $MgSO_4$, 10ml 20% (w/v) glucose, 100μl 1M $CaCl_2$ per litre were added.

Yeast were grown in complete medium (YPD), containing 1% (w/v) Bacto yeast extract, 2% (w/v) Bacto peptone, and 2% (w/v) glucose; or minimal medium (SD), 0.67% (w/v) Bacto yeast nitrogen base without amino acids and 2% (w/v) glucose. When required the following nutrients were added: histidine (20 μg/ml), leucine (30 μg/ml), tryptophan (20 μg/ml), and uracil (20 μg/ml). When solid medium was required Bacto agar was added at a concentration of 2% (w/v).

2.4 Yeast genetic techniques.

Standard techniques were used in the construction and sporulation of diploids (Sherman 1983). To test for complementation of *sec* mutations, the corresponding *sec* mutant was transformed with plasmid DNA: transformants were selected on minimal medium at 25°C, and then streaked out on minimal medium and tested for the ability to form single colonies at restrictive temperatures (either 33.5°C or 37°C).

2.5 General manipulations of DNA.

All standard techniques including restriction endonuclease cleavage, ligation, extraction with phenol, and precipitation in ethanol were carried out as described by Maniatis *et al.,* (1982). Gel electrophoresis, for visualisation of DNA fragments, was routinely carried out using 0.8% (w/v) agarose gels; the size of DNA fragments was estimated by comparison with an *Eco*RI and *Hind*III digest of phage λ DNA (see fig 2.1)

2.6 Transformation of bacterial and yeast cells.

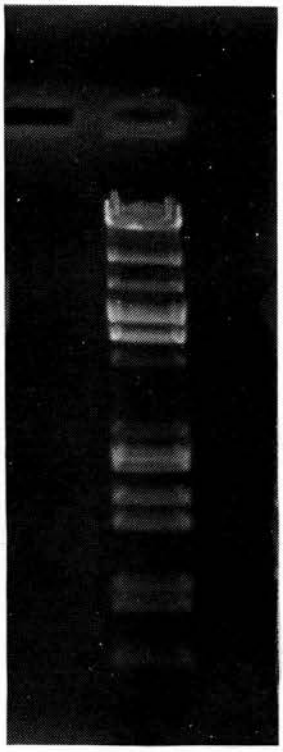
DNA was transformed into bacterial cells previously treated with $CaCl_2$ as described by Maniatis *et al.,* (1982). Yeast cells were made competent for transformation with lithium acetate, following the protocol of Ito *et al.,* (1983).

2.7 Recovery of plasmid DNA.

Plasmid DNA was recovered from bacterial cultures following the method of Birnboim and Doly (1979). For recovery from yeast cells the following method was used to prepare total DNA. A 5ml culture of transformed yeast cells was grown under selection to stationary phase, cells were harvested by centrifugation (5000rpm in a Beckman JA21 rotor for 5min) and resuspended in 0.5ml 1M sorbitol, 0.1mM EDTA pH7.5 and transferred to 1.5ml polypropylene microfuge tubes. To remove cell walls, 200µl Zymolyase 100T (2.5mg/ml) was added and the reaction incubated at 37°C for 60min. Sphaeroplasts were harvested by low speed centrifugation for 1min in a microfuge, and resuspended in 0.5ml 50mM Tris.HCl pH7.4, 20mM EDTA pH8.5. Sphaeroplasts were lysed by addition of 50µl 10% (w/v) SDS and incubation at 65°C for 30min after which 200µl 5M potassium acetate was added and the tube

Figure 2.1 Bacteriophage lambda DNA digested with *Eco*RI and *Hind*III.

DNA fragments were routinely analysed by gel electrophoresis using 0.8% (w/v) agarose gels. The size of DNA fragments was estimated by comparison to the fragments of bacteriophage lambda DNA released in an *Eco*RI and *Hind*III digest. These fragments are 21.7, 5.24, 5.05, 4.21, 3.41, 1.98, 1.90, 1.57, 1.32, 0.93, 0.84, 0.58, and 0.14kb.



placed on ice for 60min. Precipitated material was removed by high speed centrifugation in a microfuge, and the cleared supernatant decanted into a fresh microfuge tube. An equal volume of isopropanol was added to this supernatant and the tube incubated at room temperature for 5min. Precipitated nucleic acids were collected by a 10sec high speed centrifugation in a microfuge, and were resuspended in 20µl TE (10mM Tris, 0.1mM EDTA pH 8.0) buffer. *E. coli* 5K cells were transformed with approximately 500ng of this preparation, transformants being selected on L agar plates, supplemented with antibiotics as described above.

2.8 Southern hybridisation.

Transfer of DNA fragments from agarose gels to nitrocellulose filters, and the subsequent hybridisation of α-[^{32}P]radiolabelled probes to the filter, were carried out using the method of Southern (1975).

2.9 Northern hybridisations.

i) Extraction of RNA from *S. cerevisiae*:

Total RNA was prepared from DBY746 transformed with pEDB16. A 200ml culture was grown under selection to a cell density of $2x10^7$ cells per ml (mid-log phase), at which point the cells were harvested by centrifugation (5000rpm in a Beckman JA21 rotor for 5min) at 4^{o}C, and washed in one tenth volume of sterile water that had been previously treated with DEPC. The cells were again harvested by centrifugation (as above) and resuspended in 4ml guanadinium thiocyanate solution (4M guanadinium thiocyanate, 0.5% (w/v) sodium N-laurylsarcosinate, 25mM sodium citrate pH7.0, and 0.1 M ß-mercaptoethanol) and transferred to a 30ml siliconised glass tube. Cells were broken by vigorous vortexing for 30sec followed by cooling on ice for 30sec; this cycle was repeated five times. Protein was

extracted from the cell debris by several phenol-chloroform treatments, until no residual material appeared at the interface following centrifugation. RNA was precipitated from the aqueous phase at -20°C, by the addition of 0.025 volumes 1M acetic acid and 0.75 volumes ethanol. The precipitate was collected by high speed centrifugation in a microfuge, resuspended in 0.4ml DEPC-water, and extracted once more with phenol-chloroform. Re-precipitated RNA was finally resuspended in DEPC-water at a concentration of 3-5mg/ml. This method of extraction yielded approximately 1mg RNA.

ii) Electrophoresis and blotting of RNA.

RNA samples were fractionated by electrophoresis through formaldehyde denaturing agarose gels. 3g of agarose were dissolved in 130ml DEPC-water by heating; on cooling to 70°C, 40ml 5x MOPS (in 1l: 20.9g MOPS, 3.4g NaOAc.$3H_2O$, 0.95g EDTA, and adjusted to pH7.0) and 33.5ml 40% (v/v) formaldehyde were added and the gel poured immediately. To prevent loss of formaldehyde, the gel was not submerged into running buffer until immediately prior to use.

Prior to loading, 4.5µl of sample (approximately 20µg) was diluted to 20µl by the addition of 2µl 5xMOPS, 10µl deionised formamide, and 3.5µl 40% (v/v) formaldehyde; and then placed at 65°C for 10min to destroy RNA secondary structure. 3µl sterile loading buffer (50% (v/v) glycerol, 1mM EDTA, 0.4% (w/v) bromophenol blue, 0.4% (w/v) xylene cyanol) was added and the samples applied to the gel immediately. Electrophoresis was carried out in 1xMOPS running buffer, at 100V for 4hr.

RNA was transferred to "Genescreen" transfer membrane (supplied by New England Nuclear) by capillary action using 20x SSC (3M NaCl, 0.3M $C_6H_5Na_3O_7.2H_2O$), and crosslinked to the membrane by baking at 80°C in a vacuum oven for at least 2hr.

Filters were prehybridised in 20ml 50% (v/v) deionised formamide, 1M NaCl, 1x "P" buffer (0.2% (w/v) BSA, 0.2% (w/v) PVP, 0.2% (w/v) Ficoll, 50mM Tris.HCl pH7.5, 0.1% (w/v) $Na_2P_4O_7.10H_2O$, 1% (w/v) SDS), and 100µg denatured salmon sperm DNA, at 42°C for 12-18hr. The probe (in a volume no more than 500µl) was boiled and added directly to the prehybridisation mix and allowed to hybridise for 16-48hr. Following hybridisation, the filter was removed and washed as follows: 2x 5min at room temperature in 2x SSC; followed by 2x 30min. at 65°C in 2x SSC, 0.5% (w/v) SDS; and finally 2x 30 min. at room temperature in 0.1x SSC. Filters were subsequently exposed at -70°C with intensifying screens, using Kodak X-OMAT film.

2.10 Preparation of radiolabelled DNA fragments.

Two methods of radiolabelling DNA fragments were routinely used in this work The 4.3kb *Hind*III-*Bam*HI fragment of pEDB1 and the 1.1kb *Hind*III fragment of YIp30 carrying the *URA3* gene (Botstein *et al.*, 1979) were purified from agarose gels following the method described by Maniatis *et al.*, (1982). 25ng of purified material was radiolabelled following the method of Feinberg and Vogelstein (1982): DNA synthesis was primed on denatured DNA by random hexadeoxynucleotides (obtained from Pharmacia) in the presence of the Klenow fragment of *E. coli* DNA polymerase I and $\alpha[^{32}P]$-dATP. Under these conditions DNA was labelled to approximately $2x10^9$ cpm/µg.

Alternatively, the 2.9kb *Hind*III-*Xho*I of pYI51 was cloned into the *Hind*III-*Sal*I restriction sites of bacteriophage M13mp18 and M13mp19 to form pHX18 and pHX19 respectively. Single stranded DNA was prepared as for DNA sequencing (described below), and DNA synthesis primed by "hybridisation probe" primer (obtained from Pharmacia) in the presence of the Klenow fragment of *E. coli* DNA polymerase I and $\alpha[^{32}P]$-dATP, following the protocol of Hu and Messing (1982).

2.11 Transposon mutagenesis.

The transposon Tn5 used in this study is carried by a phage lambda vector. The phage carries mutations in the *cI*, *O*, *P*, and *rex* genes (Lb_{221}, *Oam*, *Pam*, *cI857*, *rex*::Tn5) which prevent it from either replicating or lysogenising when present in a sup^o background. In such a cell the phage lies dormant, and thus any kanamycin resistant colonies isolated represent transposition of a Tn5 molecule; transposon mutagenesis experiments were therefore carried out using *E. coli* strain 159 as a host.

i) Preparation of phage lysate.

A 5ml culture of *E. coli* 5K was grown for 18hr in BBL broth (1% (w/v) BBL trypticase, 0.5% (w/v) NaCl), 1% (w/v) maltose, and 10mM $MgCl_2$. Approximately 100µl of this was mixed with 100µl λ::Tn5 (10^2-10^3 pfu/ml) and incubated at room temperature for 10min. Following this, 6ml of "top" BBL agar (as BBL broth plus 0.65% (w/v) agar) was added and the mixture was overlayed onto a BBL plate (as BBL broth plus 1.5% agar). Plaques were observed after overnight incubation at 37°C. A single plaque was removed and added to 200µl phage buffer and 5µl chloroform. This mixture was shaken at 37°C for 30min to remove chloroform. A fresh culture of *E. coli* 5K was infected and plated as above, although incubation at 37°C was only allowed for 5hr. The "top" agar containing the phage was broken and collected in a tube, and subsequently centrifuged (6000rpm in a Beckman JA21 rotor for 20min.). The supernatant was removed and mixed with 200µl chloroform, to remove surviving bacteria. The phage titre was determined by an infection of *E. coli* 5K, a titre above 10^{10} pfu/ml being sufficient for use. Prior to use the supernatant was shaken at 37°C for 2hr to remove the chloroform.

ii) Tn5 directed mutagenesis.

E. coli 159 was transformed to Ap^R with pEDB16. A 5ml culture of this strain was grown under selection, in the presence of 0.2% (w/v) maltose, to mid log phase (A_{600}-0.5). The cells were harvested by centrifugation (5000rpm in a Beckman JA21 rotor for 5min) and resuspended in 2.5ml phage buffer (2mM KH_2PO_4, 5mM Na_2HPO_4, 8.5mM NaCl; this solution was autoclaved and on cooling 100µl 1M $MgSO_4$, 10µl 1M$CaCl_2$, 100µl 1% (v/v) Gelatine per 500ml was added). After removing chloroform from the phage lysate, 1ml was mixed with 1ml bacterial cells, to produce a multiplicity of infection of approximately 10. The infection was allowed to proceed for 10min at room temperature, after which 4ml of LB was added and the mixture incubated at 42°C for a further 10min. The cells were harvested by centrifugation (5000rpm in a Beckman JA21 rotor for 5min) and resuspended in 100µl LB, and then plated onto L agar plates containing ampicillin and kanamycin. Many colonies were observed following overnight incubation at 37°C, which were washed of the plates, and the resulting cell suspension used to inoculate a 50ml culture. This culture was grown under selective conditions to stationary phase and plasmid DNA extracted; approximately 2µg of this preparation was used to transform *E. coli* 5K to Km^R. Plasmid DNA from individual transformants was screened for the nature of the Tn5 insertion they contained, using restriction endonuclease digests.

2.12 DNA sequence analysis.

i) Construction of a set of *Bal*31 generated deletions in the *SEC1* gene.

The 5.6kb *Hind*III-*Pst*I fragment of pYI51 was cloned into the *Hind*III-*Pst*I sites of Bluescribe M13+ (see Stratagene catalogue 1988) to form plasmid pME1. Approximately 20µg of pME1 was linearised by cleavage at the *Xho*I site and then treated with *Bal*31 exonuclease using standard buffers and conditions (Maniatis *et*

al., 1982) Linearised pME1 (20μg) was resuspended in 100μl water to which 100μl "Bal31 reaction buffer" (40mM Tris.HCl pH8.0, 1.2M NaCl, 24mM $MgCl_2$, 24mM $CaCl_2$, 2mM EDTA) and 15 units *Bal*31 exonuclease were added; the reaction was incubated at 30°C. To obtain a number of different deletion endpoints, aliquots (20μl) were removed at different time points (T= 0,2,4,6,8,10,15,25, and 30min) and the reactions terminated by addition of 4μl 0.5M EGTA pH7.5. Using these conditions approximately 70bp were deleted from each end of the DNA molecules per minute. The different aliquots were pooled and subjected to a filling-in reaction using the Klenow fragment of DNA-polymerase I to ensure that the ends of DNA molecules were flush. Finally, DNA was cleaved with *Sma*I and the resulting fragments religated together. This DNA was used to transform *E. coli* NM522 to Ap^R, and then individual transformants screened for the nature of the plasmid they contained. The various deleted derivatives that were isolated are shown in fig 2.2. For sequencing, each deletion was transferred to bacteriophage M13mp19 (Messing 1983) as a *Hind*III-*Eco*RI fragment, or in the case of pMEΔ0 as an *Hind*III-*Xho*I fragment ligated into the *Hind*III-*Xho*I restriction sites.

ii) Preparation of single stranded DNA.

A single plaque was used to infect 20μl of exponentially growing *E. coli* NM522 cells, in a 30ml polypropylene tube. After 5min at room temperature, 1.5ml of 2x TY (1.6% (w/v) Bacto tryptone, 1% (w/v) Bacto yeast extract, 0.5% (w/v) NaCl) growth medium was added and the cultures incubated at 37°C for 8-18 hr. During incubation the cultures were shaken vigourously to provide adequate aeration.

The cultures were transferred to 1.5ml polypropylene microfuge tubes, and the cells harvested by high speed centrifugation in a microfuge. Supernatants were decanted into fresh microfuge tubes and single stranded DNA prepared from these stocks. DNA was precipitated by addition of 200μl 20% (w/v) PEG6000, 2.5M NaCl solution to

Figure 2.2 **Bal31 generated deletions in the SEC1 gene.**

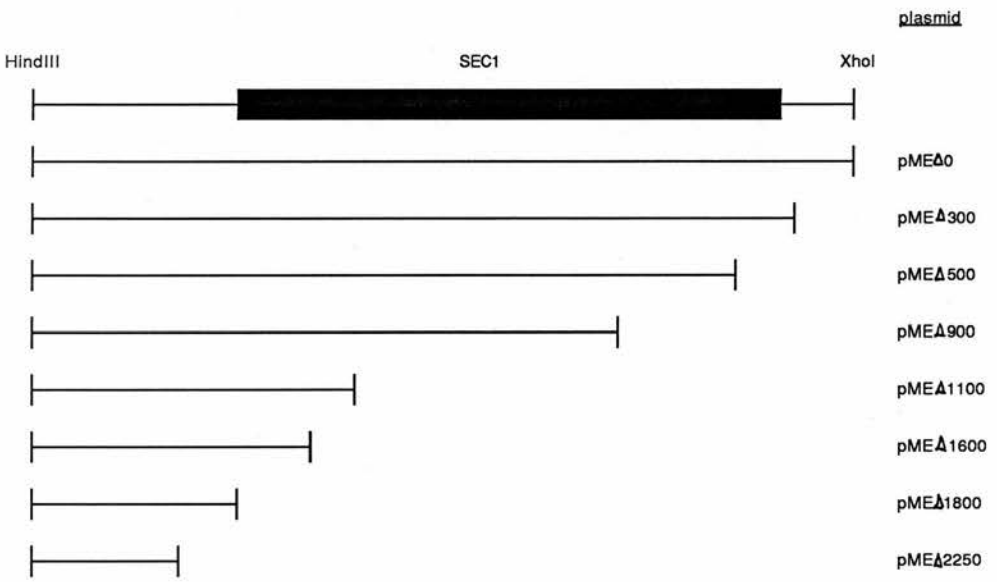


Diagram showing Bal31 deletion endpoints, see text for further details.

900µl supernatant. After 60min at room temperature the DNA precipitate was collected by high speed centrifugation in a microfuge, and the pellet resuspended in 200µl TE buffer. This was extracted two times by the addition of an equal volume of a 1:1 phenol-chloroform solution, after which DNA was precipitated out of the aqueous phase in the presence of one tenth volume 3M NaOAc pH5.5 and two volumes ethanol at -20°C. Precipitated DNA was collected by centrifugation and resuspended in 20µl TE buffer, 5µl of which was used in a typical sequencing experiment.

iii) Sequencing of DNA: DNA molecules were sequenced using the dideoxy chain termination protocol of Sanger *et al.*,(1977), in the presence of [^{35}S]-dATP (Williams *et al.*,. 1986). Sequencing reactions were electrophoresed on 6% acrylamide gels (0.4mm thickness) containing 8M urea.

2.13 Coupled *in vitro* transcription and translation of DNA molecules.

Plasmid DNA was extracted from bacterial cells by the method of Birnboim and Doly (1979), and further purified by centrifugation in a CsCl density gradient as described by Maniatis *et al.*, (1982). Approximately 5µg of DNA was transcribed and translated *in vitro* by introduction into a bacterial cell-free system following the protocol of Zubay (1973), using a kit obtained from Amersham International plc. During the reaction, newly synthesised proteins were radiolabelled by incorporation of [^{35}S]-methionine, and were then visualised by gel electrophoresis and fluorography as described below.

2.14 Gel electrophoresis of proteins.

Proteins were electrophoresed through polyacrylamide gels following the basic procedures and buffer system of Laemmli (1970). The constitution of the various buffers and solutions is given in table 2.2; 10% (w/v) acrylamide separating gels

with a 5% (w/v) acrylamide stacking gel were routinely used. Electrophoresis was usually carried out using the Hoeffer "mighty small" apparatus. Gels were cast between a glass plate and an alumina back plate (10x8cm), seperated by two PVC vertical spacers, held together by clips and sealed with 1% (w/v) molten agarose. Proteins were electrophoresed at a constant current of 40mA, with the cooling system in operation. Alternatively, larger slab gels (18x14cm) were used, which were electrophoresed at a constant current of 10mA for 18hr.

When the dye front had reached the bottom of the gel the power supply was disconnected and the gel removed. For visualisation, the gel was first immersed in fixing solution (1% (v/v) acetic acid, 20% (v/v) methanol) for 30min with gentle agitation. Protein bands were then stained with 0.25% Coomassie Brilliant Blue P250 dissolved in 50% (v/v) methanol, 7.5% (v/v) acetic acid for 10min. Gels were destained by agitation in 10% (v/v) methanol, 7% (v/v) acetic acid, with a piece of polyurethane foam to absorb free dye.

For fluorography, radioactive gels were dehydrated by successive washes in dimethylsulphoxide, followed by impregnation with PPO exactly as described by Bonner and Laskey (1974). Gels were then dried onto a sheet of Whatman 3MM chromatography paper, and exposed at -70°C using Kodak X-OMAT film.

2.15 Purification of a cro-ß-galactosidase-SEC1p fusion protein.

A 1l culture of *E. coli* NF1, transformed to Ap^R by pEXS1 was grown at 30°C to A_{600}=0.3. The culture was then incubated at 42°C for a further 5hr to induce expression of the fusion protein. Cells were harvested by centrifugation and washed in 300ml 0.1M Tris.HCl pH7.5, and then resuspended in 10ml 0.1M Tris.HCl pH7.5 per g wet weight of cells. Cells were lysed by addition of lysozyme to this suspension, to a final concentration of 1mg/ml. The cell suspension was incubated on ice for

Table 2.2 Solutions and buffers used in polyacrylamide gel electrophoresis.

A. Seperating gel buffer:

0.75M Tris.HCl pH8.8, 0.2% (w/v) SDS

B. Stacking gel buffer:

0.25M Tris.HCl pH6.8, 0.2% (w/v) SDS

C. Acrylamide solution:

44% (w/v) acrylamide, 0.8% (w/v) N;N'-methylene-bis-acrylamide (bis)

D. Electrophoresis buffer:

0.125M Tris, 0.192M glycine, 0.1% (w/v) SDS

(gives pH8.3 without adjustment)

E. Sample buffer:

0.0625M Tris.HCl pH6.8, 20% (w/v) glycerol, 4% (w/v) SDS, 5% (w/v) ß-mercaptoethanol

20min, and then sonicated for four periods of 30sec using a large sonication probe. Throughout sonication the sample was kept on ice to prevent excess warming. Cell debris was removed by centrifugation (1000rpm in a Beckman JA21 rotor for 5min), and inclusion bodies harvested from the supernatant by further centrifugation (15000rpm in a Beckman JA21 rotor for 20min). Purified inclusion bodies were washed twice in 25ml 0.1M Tris.HCl pH7.5, 0.1% (v/v) Triton X-100; and finally resuspended in 1ml per g cells 100mM Tris.HCl pH8.0, 150mM NaCl, 6mM ß-mercaptoethanol, 1% (w/v) SDS, 1% (v/v) Triton X-100. Some difficulty was encountered in getting all the material to dissolve, which was overcome by a 10min. incubation at 65°C. A sample of this material (approximately one third) was further purified by passage over a 100ml G150 Sephadex column, in 100mM Tris.HCl pH8.0, 150mM NaCl, 6mM ß-mercaptoethanol buffer. Fractions from this column were analysed by SDS-PAGE, and the desired fractions pooled and stored at 4°C.

2.16 Immunisation of rabbits with purified fusion protein.

Approximately 300µg of purified inclusion bodies were emulsified with an equal volume of complete Freund's adjuvant, using a Sorvall omni-mixer with a microattachment. Emulsified material was taken up into a 1ml syringe, avoiding air bubbles, and injected into rabbits with a 19 gauge sterile needle. Two young female New Zealand White rabbits were each immunised with this amount of material by sub-cutaneous injection, into at least 8 different sites over the back of the rabbit. This was followed 5 weeks later by a subcutaneous booster of 250µg of protein emulsified with incomplete Freund's adjuvant as described above. Both rabbits were boosted 3 times, at intervals of 2 weeks. On the seventh day following each boost, a 30ml sample of blood was taken from each animal, and a clot allowed to form by standing the blood at room temperature for 2hr and then at 4°C for a further 18hr. The serum was decanted and centrifuged at 5000rpm in a Beckman rotor (type

JA21) for 10min, and then filter sterilised by passage through a 0.22μm millipore filter and stored at 4°C.

Chapter Three.

Isolation of a fragment of yeast genomic DNA the can complement the *sec1-1* mutation.

3.1 Introduction.

The studies presented in this chapter describe the isolation of a genomic DNA fragment that is capable of complementing the *sec1-1* mutation at 37°C. A library of random *S. cerevisiae* genomic DNA fragments was obtained from J. Hicks (Nasmyth and Tatchell 1980). A detailed protocol describing the formation of this gene bank can be found in the above reference but briefly, genomic DNA was cleaved with *Sau*3A under conditions that resulted in partial digestion. DNA fragments with an approximate size of 10kb were purified and cloned into the *Bam*H1 site of YEp13 (see fig1.1, Broach *et al.*, 1979). For propagation in bacterial strains the plasmid has both a bacterial origin of replication and genes conferring resistance to ampicillin (Ap^R) and tetracycline (Tc^R, cloning into the *Bam*H1 site disrupts this gene); the plasmid also contains the *S. cerevisiae* gene encoding ß-Isopropylmalate dehydrogenase (*LEU2*, Andreadis *et al.*, 1984) and an origin of replication taken from the endogenous yeast 2µm plasmid (Broach 1981), allowing it to be maintained and selected for in *S. cerevisiae* strains. The plasmid is present in multicopy - an approximate copy number of 50-70 plasmids per cell is usual. The *LEU2* gene allows transformed cells to be selected by growth on minimal medium in the absence of leucine, after which these strains can be screened for the gene(s) of interest.

3.2 Isolation of a recombinant clone that can complement the *sec1-1* mutation.

Approximately 10µg of the genomic library DNA was used to transform yeast strain ABY12 (*sec1-1, leu2-3,112, his3-11,15*) to leucine prototrophy. Transformed cells were selected by growth on minimal medium, supplemented with histidine, at 26°C. Approximately 10,000 transformants were recovered after 48hr of growth under these conditions. The transformants were washed off the plates, with sterile SD, to form a suspension with an approximate cell density of $5x10^8$ cells/ml. Cells were subsequently plated onto complete medium and incubated at 37°C for 20hr in

order to select for temperature resistant strains. Many colonies were recovered, ten of which were picked for further analysis. The cosegregation of the *LEU2* gene and the *sec1-1* complementing gene was tested by growth, through several generations on complex medium at 26°C; all colonies that had lost the ability to grow in the absence of leucine had also lost the ability to grow at 37°C, demonstrating complete linkage of the two markers. Total DNA was prepared from temperature resistant yeast cells and used to transform *E.coli* 5K to Ap^R. Individual bacterial transformants were screened for the nature of the plasmid they contained; only one species of plasmid was observed, which has been termed pEDB1. When reintroduced into ABY12, this plasmid rendered the host cells temperature resistant. Evidence is presented in section 3.4 and chapter 5 that the gene responsible for *sec1-1* complementation is the authentic *SEC1* gene, and therefore for ease of writing the "cloned gene" is referred to as the *SEC1* gene from now on.

3.3 Subcloning of the *SEC1* gene.

A restriction map of the genomic fragment of DNA present in pEDB1 for the enzymes *Bam*HI, *Eco*RI, *Hind*III, and *Pst*I has been compiled and is shown in figure 3.2. The DNA fragment is 10.6kb in length and contains numerous recognition sites for the enzymes mentioned above. The pattern of *Hind*III cleavage sites immediately suggested a strategy for subcloning the *SEC1* gene. Plasmid pEDB1 was cleaved with *Hind*III and the resulting mixture of fragments used in a ligation reaction. A number of pEDB1 derivatives were obtained (pEDB14, pEDB15, pEDB16, and pEDB18), which as detailed in figure 3.3. All the plasmids carry the 4.3kb *Hind*III-*Bam*HI fragment, either alone (pEDB16) or in combination with a second *Hind*III fragment. The four derivatives were tested for the ability to complement the *sec1-1* lesion in ABY12 following a similiar protocol to that described above. All four plasmids were observed to support growth of ABY12 at 37°C indicating that the gene responsible for complementation is located within the 4.3kb *Hind*III-*Bam*HI fragment.

The restriction map for this region has been extended to include cleavage sites for many more common enzymes, and is shown in Fig 3.4. The *Xho*I site is unique within this region and lies towards the centre of the fragment. To determine if the *SEC1* gene spanned this site, the 4.3kb*Hind*III-*Bam*HI fragment was cloned into YEp213 (Broach 1983), to form pEDB213 thereby making the *Xho*I site unique, after which it was disrupted by insertion of a 1.2kb *Sal*I fragment carrying a Km^R gene. This had no effect on the capability of the fragment to complement the *sec1-1* mutation (data not presented), consistent with the *SEC1* gene lying to one side of the *Xho*I site.

3.4 Demonstration that the cloned gene can complement the *sec1-1* mutation when present in single copy only.

The strategy of isolating *S. cerevisiae* genes from genomic libraries made in multicopy vectors has proven successful on many occasions (e.g see Nasmyth and Reed 1980, Nasmyth and Tatchell 1980, Dietzal and Kurjan 1987). However, any positive results have to be treated with caution because on numerous occasions genes have been isolated, other than the wild type gene, that are capable of rescuing the mutant strain - a phenomenon termed "mass action suppression" (e.g. see Dietzal and Kurjan 1987). The final experiment presented in this section provides preliminary evidence that the cloned gene is the authentic *SEC1* gene. I have utilised a second class of *E.coli / S. cerevisiae* shuttle vector, termed a YIp plasmid, which lacks the elements of yeast DNA which would allow it to replicate independently of the genome. This type of plasmid transforms yeast cells by integrating into the host genome, via homologous recombination, so that it can be replicated during host DNA synthesis. Such integration events are infrequent, resulting in the plasmid being present in transformed cells at low copy number. Using this approach it has been possible to demonstrate that the cloned gene is capable of complementing the *sec1-1* mutation when present in single copy only.

The 4.3kb *Hin*dIII-*Bam*HI fragment was cloned into the respective sites of YIp5 (see figure 3.5, Struhl *et al.,* 1979), to form plasmid pYI51. This plasmid carries the yeast *URA3* gene as a selectable marker (Rose *et al.,* 1984), so that when pYI51 is used to transform yeast cells it is possible for the plasmid to integrate into the genome, via homologous recombination, at either the *URA3* locus or the locus represented by the 4.3kb *Hin*dIII-*Bam*HI fragment. Integration can be targeted, however, by cleavage of the plasmid within one of the yeast sequences, to produce a linear molecule whose ends are highly recombinogenic. To direct integration to the chromosomal locus corresponding to the 4.3kb *Hin*dIII-*Bam*HI fragment, approximately 10μg of pYI51 was linearised by cleavage at the *Xho*I site, and used to transform MEY12 to uracil prototrophy. Following 48hr growth on minimal medium at 26°C more than 200 transformants were obtained of which fifteen were taken for further analysis. The transformants were patched onto selective medium and grown at 26°C for 48hr, after which they were replica plated onto complete medium and grown at 37°C. All fifteen transformants grew at this temperature during overnight incubations, confirming that the cloned gene is capable of complementing the *sec1-1* mutation even at low copy number.

From this sample of fifteen transformants, five strains (designated MEY12X1 through to MEY12X5) were taken for further analysis. Initially, the co-segregation of the URA^+ and SEC^+ phenotypes was followed through several generations of growth on complex medium - integrated plasmids are observed to be 100% stable. The five strains were plated onto complete medium and grown at 26°C until visible colonies appeared (48hr), and then replica plated onto selective and non-selective media. All five strains showed 100% stability of the URA^+, SEC^+ phenotypes. It is possible however, for a cell to possess more than one copy of pYI51, brought about by multiple integration events (see fig 3.6). It was essential, therefore, to identify a strain which had received only a single copy of the plasmid. Integration of a plasmid at an homologous chromosomal locus results in an altered pattern of restriction fragments within that region: it is possible to utilise this characteristic and in

conjunction with Southern hybridisation experiments, determine the copy number of the integrated plasmid.

To demonstrate integration at the chromosomal locus that is homologous to the 4.3kb *Hind*III-*Bam*HI fragment, genomic DNA was prepared from the five strains (MEY12X1 through to MEY12X5), and following cleavage with *Hind*III, probed with the 4.3kb *Hind*III-*Bam*HI fragment. The model predicts two fragments will hybridise to this probe: a 9.5kb fragment corresponding to the integrated plasmid; and a fragment of at least 4.3kb corresponding to the chromosomal *Hind*III fragment carrying the *SEC1* gene. The results are shown in fig 3.7; two bands can clearly be seen on the autoradiograph, which are judged to be 9.5kb and 4.6kb respectively by extrapolation to size markers on the original agarose gel (data not shown). This indicates that the right hand flanking genomic *Hind*III site, as drawn on the map, lies approximately 300bp from the *Bam*HI site.

The plasmid copy number of the MEY12 integrants was determined by probing genomic DNA, digested with *Pvu*II, with the purified 1.1kb *Hind*III fragment from YIp30, which carries the *URA3* gene. A strain which has experienced a single integration event would be expected to produce two fragments that would hybridise to this probe: a fragment corresponding to the chromosomal *Pvu*II fragment carrying the *URA3* gene (of unknown size at this point); and a fragment of at least 7kb, corresponding to the distance from the plasmid *Pvu*II site to the nearest chromosomal *Pvu*II site on the left as shown in Fig 3.6. Strains that possess tandem integrants will produce both of these fragments, along with a third fragment of 9.5kb, corresponding to pYI51. The results are shown in Fig 3.8: all strains produce two bands of 3.5kb and at least 15kb; the smaller band is also present in DBY746 and therefore represents the genomic *Pvu*II fragment carrying the *URA3* gene, whereas the large band represents the hybrid plasmid - genomic DNA fragment. In addition three strains (MEY12X1, X3, and X5) produce a third band of 9.5kb, and thus present multiple integrants. MEY12X2 and MEY12X4 are therefore strains in which

the cloned gene complements the *sec1-1* mutation when present as a single copy integrant.

3.5 Summary

In this chapter I have described the isolation of a recombinant plasmid (pEDB1), that carries a 10.6kb fragment of yeast genomic DNA, capable of complementing the *sec1-1* mutation. Analysis by subcloning has localised the gene responsible for this complementation to within a 4.3kb *Hind* III-*Bam* HI fragment. The genomic library from which this clone was isolated was, however, constructed in a vector that is maintained in multi-copy; it is possible therefore that pEDB1 contains a mass action suppressor of the *sec1-1* mutation. To demonstrate that this is not the case I have shown that the 4.3kb *Hind*III-*Bam*HI fragment can complement the *sec1-1* mutation even when it is integrated into the genome at single copy. This evidence alone does not eliminate the possibility that a gene has been isolated that can suppress the *sec1-1* mutation when it is duplicated - this has been recently demonstrated for the *sec15-1* mutation which can be suppressed by duplication of the *SEC4* gene (Salminen and Novick 1987). Further genetic evidence has therefore been sought to confirm that the 4.3kb *Hind*III-*Bam*HI fragment carries the authentic *SEC1* gene (see chapter 5)

Figure 3.1 Map of YEp13.

YEp13 carries the genes conferring resistance to ampicillin (Ap^R) and tetracycline (Tc^R), and the origin of replication from pBR322 (Bolivar *et al.*,1977), which allow the plasmid to be maintained in *E. coli*. The plasmid also carries the *S. cerevisaie LEU2* gene and elements of the endogenous 2μm plasmid which allow it to be maintained and selected for in yeast cells. See text for further details. B (*Bam*HI), H(*Hind*III), P (*Pst*I), R (*Eco*RI), S (*Sal*I).

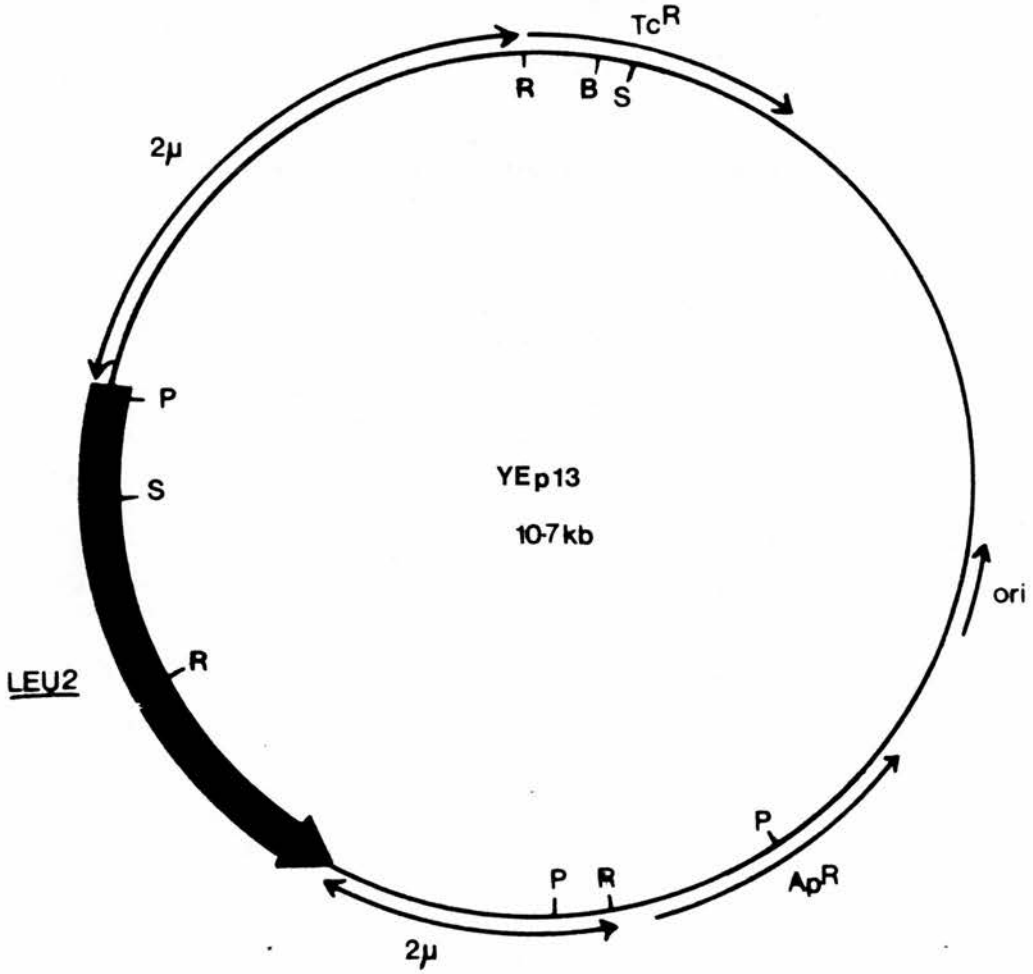
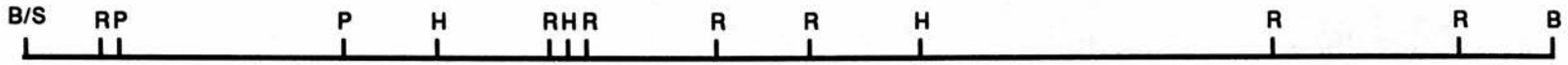

TcR
R
B
S
2μ
P
S
YEp13
10·7kb
ori
LEU2
R
P
P
R
ApR
2μ

Figure 3.2 Fragment of yeast genomic DNA carried by pEDB1.

By restriction mapping pEDB1 contains a 10.6kb fragment of yeast genomic DNA, that was generated as a partial *Sau*3A fragment and cloned into the *Bam*HI site of YEp13. During this experiment the *Bam*HI site at the right hand insert/vector DNA junction was recreated, although this does not mean that a *Bam*HI site appears in the genome at this position. The location of several restriction sites are shown: B (*Bam*HI), B/S (hybrid *Bam*HI/*Sau*3A), H (*Hind*III), P (*Pst* I), R (*Eco*RI).



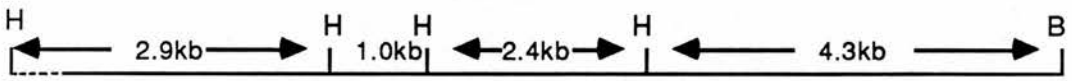
Scale: 1kb

Figure 3.3 Derivatives of pEDB1.

A. Shows a restriction map of the 10.6kb genomic fragment of DNA from pEDB16, with the *Hind*III (H) and *Bam*HI (B) restriction sites, and the size of expected fragments marked. The left *Hind*III site is actually located in YEp13 vector sequences (- - -).

B. Approximately 1μg of pEDB1 was cleaved with *Hind*III to release a large fragment of 10.6kb (a hybrid fragment containg YEp13 vector sequences and the 4.3 kb *Hind*III-*Bam*HI fragment), and three smaller fragments of 2.9kb, 2.4kb, and 1.0kb; all of which originate from the genomic fragment of DNA. These fragments were religated together and plasmids isolated which carried the 4.3kb *Hind*III-*Bam*HI fragment, either alone (pEDB16), or in association with a second *Hind*III fragment (PEDB14, pEDB15, and pEDB18). Each plasmid was introduced into ABY12 and then tested for its ability to complement the *sec1-1* mutation; all four plasmids supported growth of ABY12 at 37°C, indicating that the gene responsible for complementation was located in the 4.3kb *Hind*III-*Bam*HI fragment.

10.6kb genomic DNA fragment of pEDB1

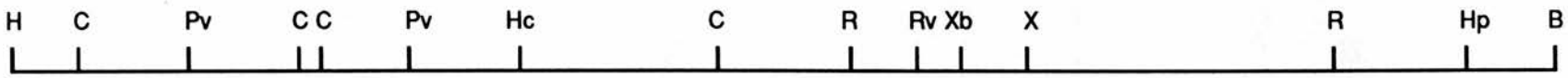


Derivatives of pEDB1

PLASMID	Fragments of pEDB1 genomic DNA carried by plasmid.	Ability to complement *sec1* lesion at 37°C
pEDB14	4.3kb HindIII-BamHI fragment and 2.4kb HindIII fragment.	+
pEDB15	4.3kb HindIII-BamHI fragment and 2.9kb HindIII fragment.	+
pEDB16	4.3kb HindIII-BamHI fragment only.	+
pEDB18	4.3kb HindIII-BamHI fragment and 1.0kb HindIII fragment	+

Figure 3.4 **Restriction map of the 4.3kb *Hind*III-*Bam*HI fragment.**

The restriction map of this region has been extended to include restriction sites for several more common enzymes. B (*Bam*HI), C (*Cla*I), H (*Hind*III), Hc (*Hinc*II), Hp (*Hpa*I), Pv (*Pvu*I), R (*Eco*RI), Rv (*Eco*RV), X (*Xho*I), Xb (*Xba*I). The *Xho*I site is also unique in the 10.6kb genomic fragment of pEDB1.



Scale 0.5kb

Figure 3.5 Map of Ylp5.

Ylp5 carries the Ap^R and Tc^R resistance genes and the origin of replication (*ori*) from pBR322, which allow the vector to be maintained and selected in *E. coli.* In addition the plasmid also carries the *URA3* gene of *S. cerevisiae* which can be used as a selectable marker in yeast strains defective for this gene. The plasmid cannot replicate episomally in yeast but can become established by recombination into the host genome. Plasmid pYI51 was formed by cloning the 4.3kb *Hind*III-*Bam*HI fragment into the *Hind*III and *Bam*HI sites of Ylp5. B(*Bam*HI), H(*Hind*III), P(*Pst*I), R(*Eco*RI).

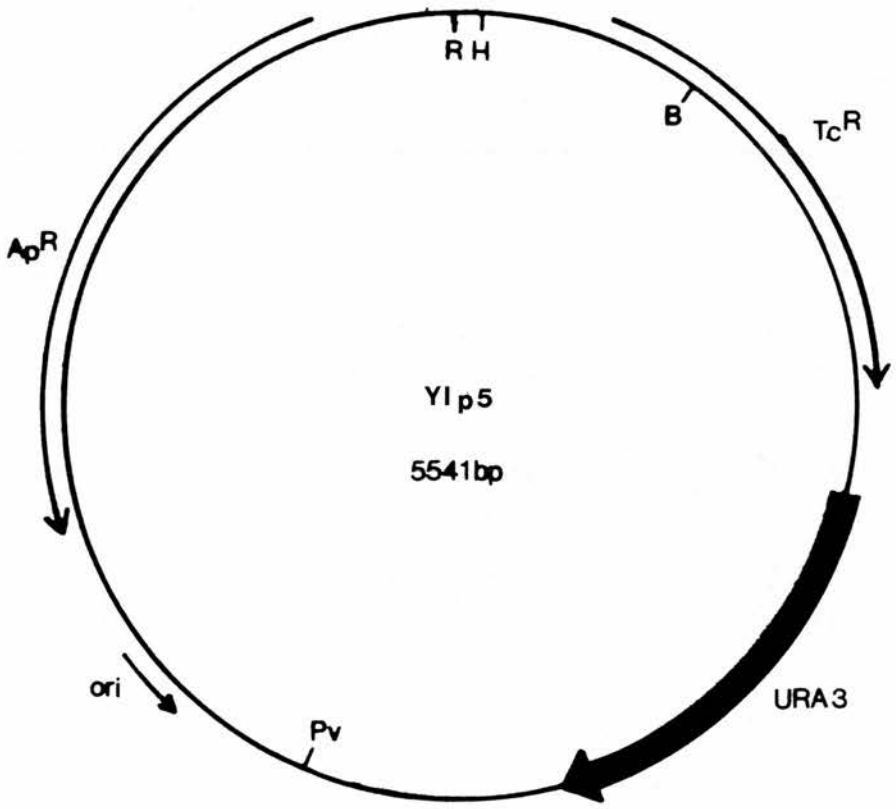

R H
B
TcR
ApR
YIp5
5541bp
ori
Pv
URA3

Figure 3.6 Schematic representation of integration of pYI51 into the host genome.

In order to become established in the cell plasmid pYI51 must integrate into the host genome by homologous recombination; in this experiment, integration was targetted to the genomic locus corresponding to the 4.3kb *Hind*III-*Bam*HI fragment, by linearisation of pYI51 with *Xho*I (A). This event was confirmed by Southern hybridisation using the 4.3kb *Hind*III-*Bam*HI fragment to probe a genomic DNA digested with *Hind*III (see text for details and fig 3.7). Multiple integration events are possible, resulting in strains with tandem integrated copies of pYI51 (B); such strains were diagnosed by probing genomic DNA, digested with *Pvu*II, with the 1.1kb *Hind*III fragment of YIp30 which carries the *URA*3 gene (see text for details and fig 3.8). Numerous restriction sites are shown, B (*Bam*HI), H (*Hind*III), and P (*Pvu*II); those sites which are bracketed represent genomic restriction sites whose exact location is not known. Ap^R and Tc^R - genes conferring resistance to ampicillin and tetracycline respectively. This diagram is not to scale.

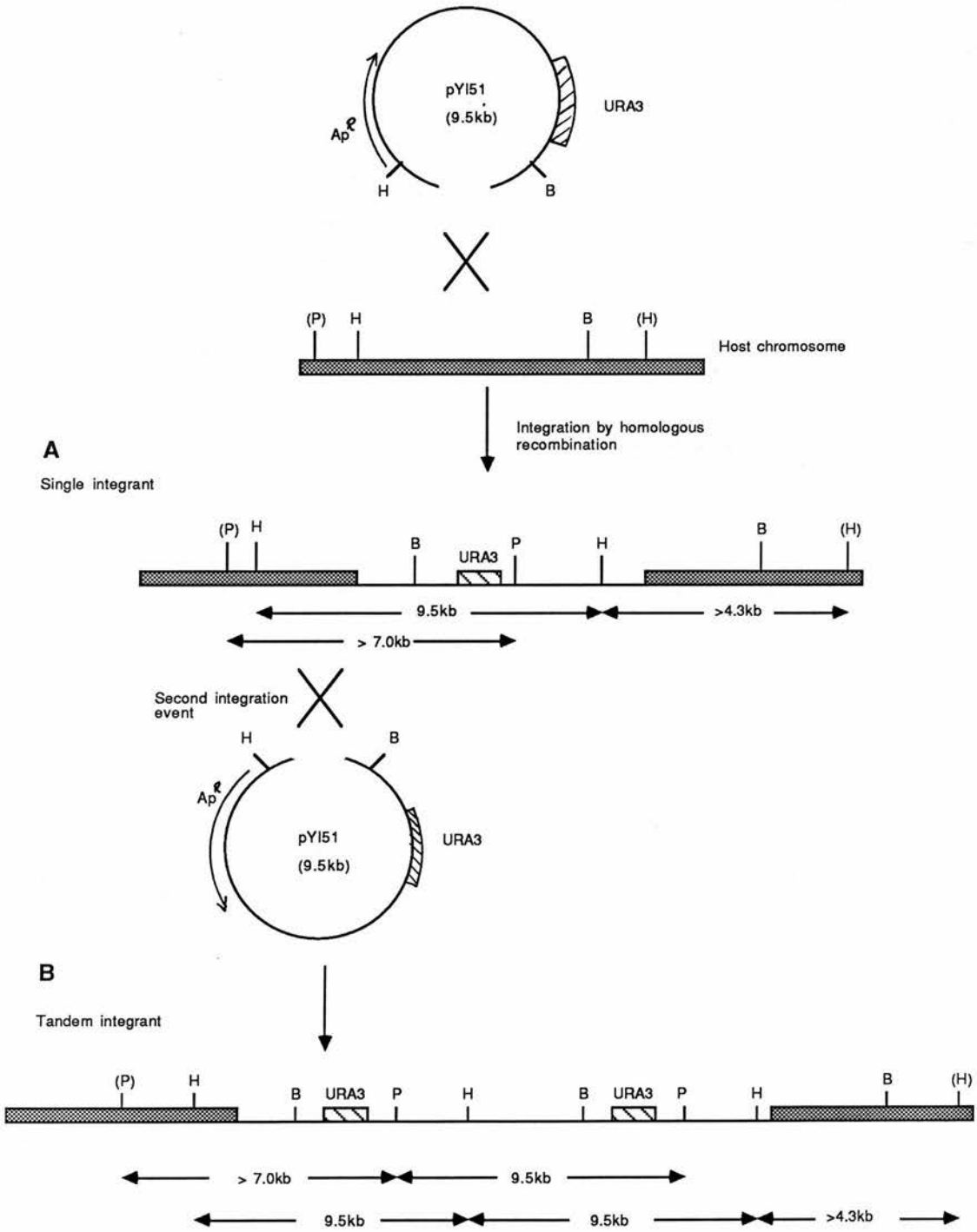

pYI51
(9.5kb)
URA3
Ap
H
B
(P)
H
B
(H)
Host chromosome
Integration by homologous recombination
A
Single integrant
(P)
H
B
URA3
P
H
B
(H)
9.5kb
>4.3kb
> 7.0kb
Second integration event
H
B
Ap
pYI51
(9.5kb)
URA3
B
Tandem integrant
(P)
H
B
URA3
P
H
B
URA3
P
H
B
(H)
> 7.0kb
9.5kb
9.5kb
9.5kb
>4.3kb

Figure 3.7 **Integration of pYI51 into the genome.**

Genomic DNA was prepared from strains MEY12X1-MEY12X5 (lanes 1 to 5 respectively), cleaved with *Hind*III and the resulting fragments separated by gel electrophoresis. DNA was transferred to nitrocellulose as described in chapter 2, and probed with the 4.3kb *Hind*III-*Bam*HI fragment. In each integrant two fragments hybridise with this probe: a fragment of approximately 9.5kb corresponding to pYI51; and a fragment of 4.6kb representing the genomic *Hind*III fragment which carries the *SEC1* gene.

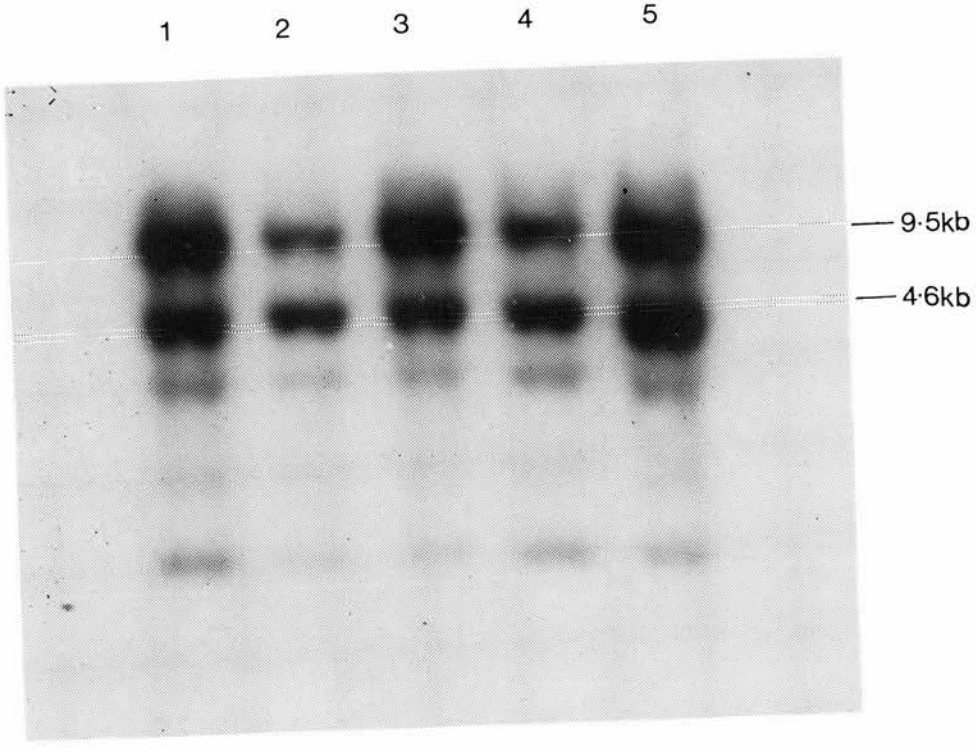
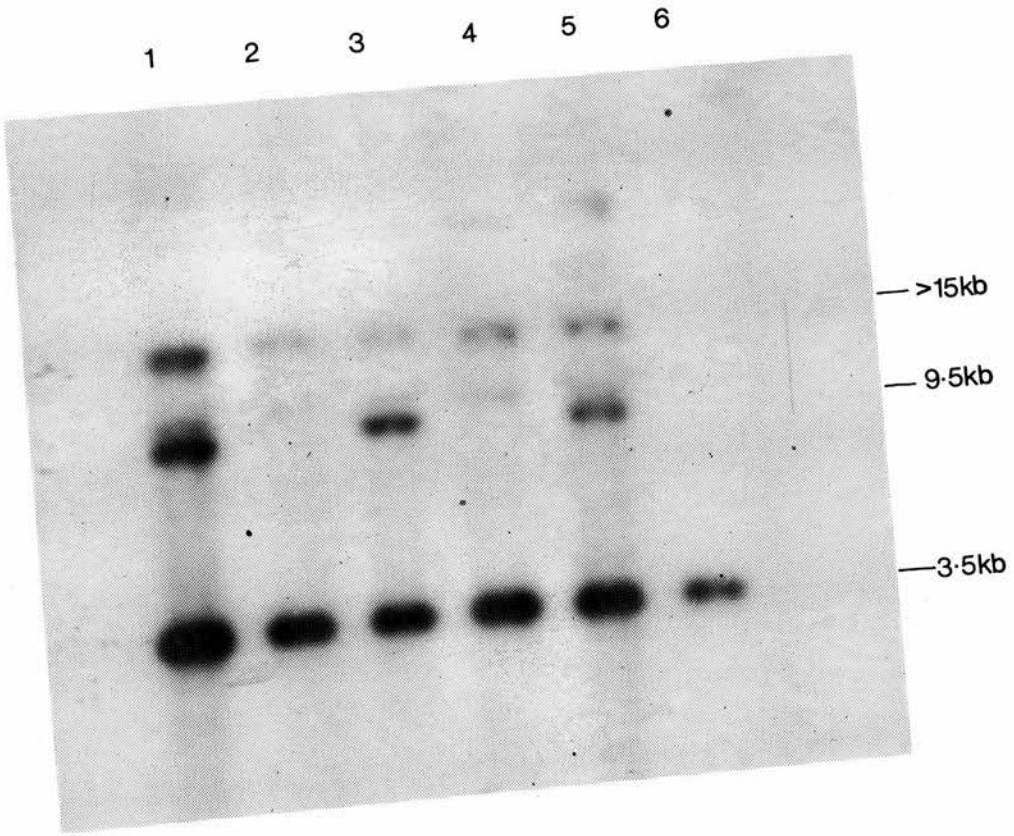

1
2
3
4
5
9·5kb
4·6kb

Figure 3.8 Copy number of integrated pYI51 molecules.

Genomic DNA was cleaved with *Pvu*II, separated by gel electrophoresis, and transferred to nitrocellulose. The filter was probed with the 1.1kb *Hind*III fragment of YIp30 which carries the *URA3* gene.

Lane 1 MEY12X1
Lane 2 MEY12X2
Lane 3 MEY12X3
Lane 4 MEY12X4
Lane 5 MEY12X5
Lane 6 DBY746

The control strain, DBY746, has only one DNA fragment that hybridises with this probe; this represents the genomic *Pvu*II fragment carrying the *URA3* gene. A maximum of three bands are seen with the strains carrying integrated copies of pYI51, whose origin is described in the text and depicted in fig 3.6. Strains MEY12X1, X3, and X5 possess all three bands and therefore have multiple copies of pYI51 integrated into the genome, whereas MEY12X2 and MEY12X4 have only two of these bands and thus contain only one integrated copy of pYI51.


1
2
3
4
5
6
>15kb
9·5kb
3·5kb

Chapter Four.

Mapping of the *SEC1* gene by transposon mutagenesis

4.1 Introduction.

In the previous chapter I have described the isolation of a 10.6kb fragment of genomic DNA that is capable of complementing the *sec1-1* mutation. Subcloning experiments have localised the gene responsible for this complementation to within a 4.3kb region of the insert. One of the main aims in this thesis was to determine the nucleotide sequence of the *SEC1* gene (described in detail in chapter 6), and therefore I set out to define the limits of the *SEC1* gene more accurately.

I chose transposon mutagenesis as the most convenient method for this mapping project. Transposons are found in both eukaryotic and prokaryotic systems. They can simply be defined as segments of DNA, that have the ability to insert into the genome of a host organism (for review see Kleckner 1981). Each transposon is a self-contained unit which encodes one or more functions that are required, in conjunction with functions specific to the host, to promote transposition. A review of the mechanism of transposition is outside the scope of this thesis, but for more detail the reader is referred to Derbyshire and Grindley (1986). The characteristics described above have been harnessed to provide a powerful experimental tool. The rationale of this study was to expose the 4.3kb *Hind*III-*Bam*HI fragment, the insert of pEDB16, to a transposon under conditions that would promote "hopping" of the transposon into this fragment. Transposition into the *SEC1* gene would abolish its ability to complement the *sec1-1* mutation, whereas transposition into regions outside the *SEC1* gene would have no such effect. The location of these two classes of insertion within the fragment will therefore mark the position of the *SEC1* gene.

In this study I have used the transposon Tn5, which is capable of inserting at high frequency into the chromosome, plasmids, and temperate phages of gram negative bacteria (for review see Berg and Berg 1983). A schematic representation of the

Tn5 molecule is shown in Fig 4.1; the molecule is 5.7kb in length, and can be roughly divided into three areas - a central region of 2.7kb which is flanked by two regions of 1.5kb. The two flanking regions, termed IS50L and IS50R, are in fact inverted repeats, of slightly different sequence, whose structure is thought to be fundamental to the mechanism of transposition (Berg *et al.,* 1980, 1982). The IS50R region also encodes two proteins: the transposase and its regulator, which are thought to catalyse the transposition event (Rothstein and Reznikoff 1981). The central region of the molecule houses the gene encoding neomycin phosphotransferase type II, which confers resistance to aminoglycoside antibiotics (Berg *et al.,* 1975); cells harboring a Tn5 molecule are therefore resistant to kanamycin and neomycin. Since the Tn5 molecule is specific to gram negative bacteria, mutagenesis of pEDB16 was carried out in *E. coli* , after which mutagenised plasmids were recovered, analysed for the nature of the insertion they contained, and then introduced into a suitable yeast strain to determine the effect of the insertion upon ability to complement the *sec1-1* mutation.

As a means of introducing the Tn5 molecule into bacterial cells, I have used a strain of phage lambda that carries the transposon. This phage also carries mutations in the *cI* gene (cI_{857}), and the *O* and *P* genes (amber nonsense) such that following infection, into a suitable host under the correct experimental conditions, the phage lies dormant since the lysogenic and lytic life cycles are blocked - stable Km^R colonies can therefore only arise as a result of transposition. The Tn5 molecule has stringent control mechanisms however (for review see Reznikoff 1982), which permit only a single transposition event to occur within the recipient cell. Therefore the appearance of a bacterial colony that is resistant to kanamycin is thought to represent a unique transposition event, thereby allowing the easy identification of many different insertions.

4.2 Tn5 mutagenesis of the 4.3kb *Hind*III-*Bam*HI fragment.

E. coli strain 159 (sup^0) was transformed to Ap^R with pEDB16. Transformed cells were used to inoculate a 5ml culture which was grown, under selection in 0.2% maltose (inducer of the lambda receptor), to mid-log phase (A_{600}-0.5). Cells were harvested and resuspended in 2.5ml of phage buffer, 1ml of which was mixed with 1ml of a phage λ::Tn5 lysate (titre of at least 10^{10} pfu/ml). The infection was allowed to proceed at room temperature for 10min, after which 4ml LB was added and the mixture incubated at 42°C for a further 10min. The cells were harvested by centrifugation and plated onto LB agar containing kanamycin and ampicillin, to select for mutagenised derivatives of pEDB16. Many colonies were observed following overnight incubation at 37°C; these were washed off the plates and the resultant cell suspension used to inoculate a larger scale culture from which plasmid DNA was prepared (this procedure segregates Tn5 insertions into plasmid DNA from insertions in the host chromosomal DNA) . Approximately 1μg of this preparation was used to transform *E. coli* strain 5K to Ap^R/Km^R and the resultant transformants were screened for the nature of the insertion they contained.

The insertion of a Tn5 molecule into pEDB16 results in drastic changes in the restriction pattern around the point of insertion. It is therefore possible to determine the position of the Tn5 molecule by restriction digest analysis. In this study I have used three enzymes, namely *Bam*HI, *Eco*RI, and *Hind*III, for this purpose. In total I have analysed plasmids from 67 Ap^R, Km^R bacterial colonies. Plasmids were first digested with *Eco*RI; when this enzyme is used to cleave pEDB16, a characteristic profile of restriction fragments is produced, see fig 4.2. The position of insertion of a Tn5 molecule can be easily localised since the transposon does not possess any *Eco*RI cleavage sites, and thus the pEDB16 *Eco*RI fragment housing the Tn5 molecule is observed to increase in size by 5.7kb (eg. see fig 4.2). Of the 67 plasmids analysed in this way, 12 were found to possess Tn5

insertions within the 2.5kb or 1.2kb *Eco*RI fragments of pEDB16. These plasmids (pTN) were taken for further analysis.

The precise position at which the transposon had inserted into pEDB16 was determined by further restriction analysis using the enzymes *Bam*HI and *Hind*III. For both of these enzymes there is only a single cleavage site in pEDB16, while Tn5 has one *Bam*HI site and two *Hind*III sites (see fig 4.1). In the mutagenised plasmids novel restriction fragments are therefore produced, the size of which can be used to accurately map the site of Tn5 insertion. I have presented the raw restriction data for only one Tn5 insertion (pTN64, see fig 4.3 and the corresponding legend for full description), as an example of the procedure that was used to map all twelve insertions. Having mapped the position of the Tn5 insertions in this way, I determined the ability of each plasmid to complement the *sec1-1* mutation (see fig 4.4). Each pTN plasmid was introduced into ABY12; by transformation, with selection for growth, at 26°C, on minimal medium in the absence of leucine. Wherever it was possible ten transformants for each plasmid were taken for further analysis; in those cases where plasmids transformed poorly the maximum number of colonies possible were analysed. Each colony was patched onto selective medium and grown at 26°C for at least 20hr, and then replica plated onto complete medium and grown at either 26°C or 37°C overnight. The results are presented in fig 4.5; of the twelve plasmids, four had lost the ability to complement the *sec1-1* mutation (pTN18, pTN26, pTN57, and pTN64). In the cases of pTN18, pTN57, and pTN64 all the transformants tested were unable to grow at 37°C; in the case of pTN26 however, three of ten transformants were viable at the restrictive temperature. This viability may be a result of recombination between the *sec1-1* chromosomal gene and the Tn5 mutagenised *SEC1* gene thereby producing a functional *SEC1* gene. These four insertions can therefore be used to mark the position of the *SEC1* gene within the 4.3kb *Hind*III-*Bam*HI fragment. In addition, the Tn5 insertions present in pTN2 and pTN7 are located no more than 200bp

outside this region and yet still retain the ability to complement *sec1-1* mutation, and thus it was possible to map the *SEC1* gene within a 1.8kb region of DNA (Fig 4.4).

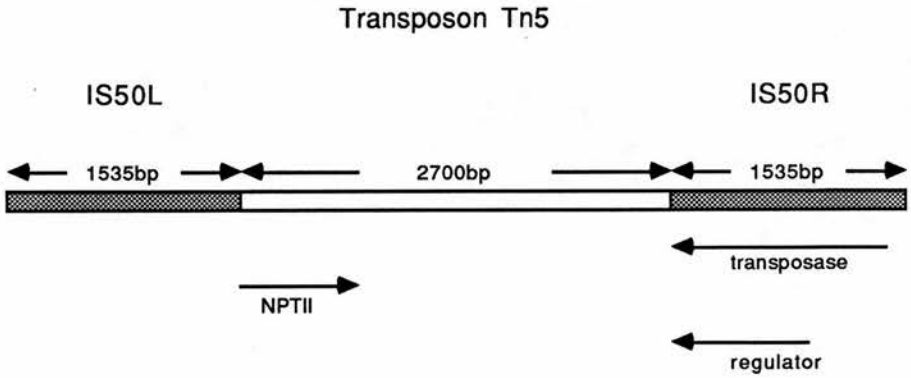
4.3 Summary.

I have used the transposon Tn5 to mutagenise plasmid pEDB16 - a derivative of pEDB1 which carries only the 4.3kb *Hind* III-*Bam* HI fragment. In total I have screened 67 pEDB16-derived plasmids which contain Tn5 insertions. By restriction analysis, twelve plasmids were found to contain insertions within the 4.3kb *Hind*III-*Bam*HI fragment. When these plasmids were subsequently tested for their ability to complement the *sec1-1* mutation, four plasmids (pTN18, pTN26, pTN57, and pTN64) were observed to be incapable of supporting the growth of ABY12 at 37°C. These four plasmids harbor Tn5 insertions within the *SEC1* gene; restriction mapping has shown that these insertions span a region of 1.6kb to the left of the *Xho*I site. Other plasmids, namely pTN2 and pTN7, harbour Tn5 insertions that lie no more than 200bp outside this region yet still retain the ability to complement *sec1-1* mutation. The *SEC1* gene has therefore been located to a 1.8kb region of DNA, located to the right of the *Xho*I site. It is possible however, for the *SEC1* ORF to extend outside this region since the insertion of a Tn5 molecule into a region of the gene that encodes a dispensable portion of the protein, would not have any effect on its ability to support growth of a *sec1-1* mutant at 37°C.

Figure 4.1 Organisation of the transposon Tn5.

Diagram A is a schematic representation of the Tn5 molecule. The shaded regions (IS50L and IS50R) are inverted repeat segments. The positions of the three open reading frames encoding the transposase, the transposase regulator, and the neomycin phosphotransferase are indicated. Diagram B shows a simple restriction map of the Tn5 molecule, with several restriction sites marked: B (*Bam*HI), H (*Hind*III), S (*Sal*I), X (*Xho*I).

A


Transposon Tn5
IS50L
IS50R
1535bp
2700bp
1535bp
transposase
NPTII
regulator

B

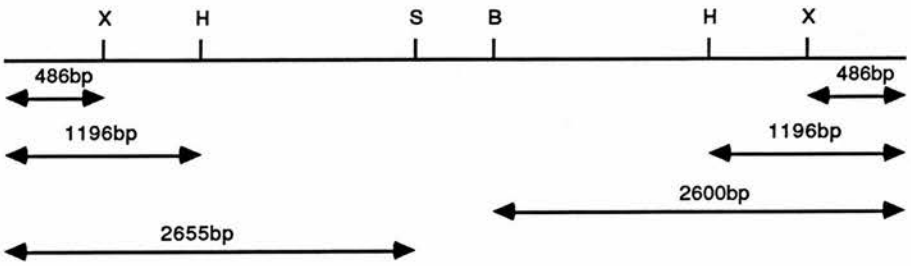

X
H
S
B
H
X
486bp
486bp
1196bp
1196bp
2600bp
2655bp

Figure 4.2 Localisation of Tn5 insertions in pEDB16 mutagenised plasmids.

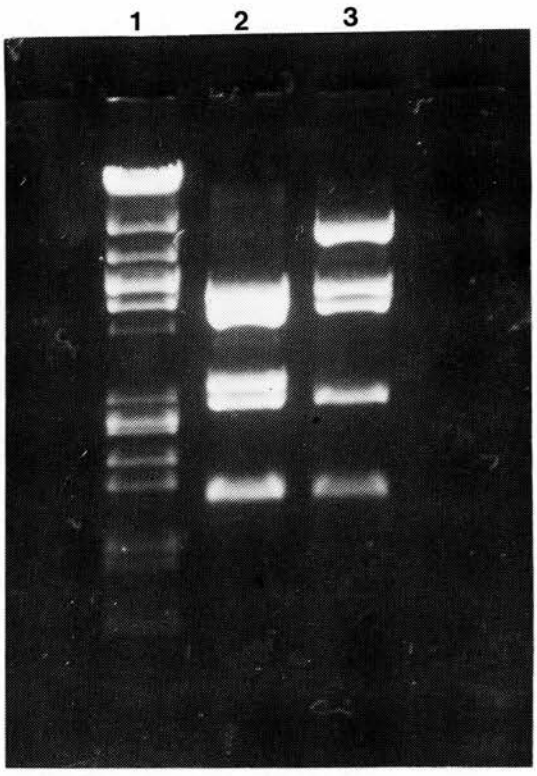
The transposon Tn5 does not have any *Eco*RI cleavage sites, and therefore insertion of Tn5 into pEDB16 results in one of the *Eco*RI fragments increasing in size by 5.7kb. Shown in diagram A are *Eco*RI restriction digests of plasmids pEDB16 and pTN64.

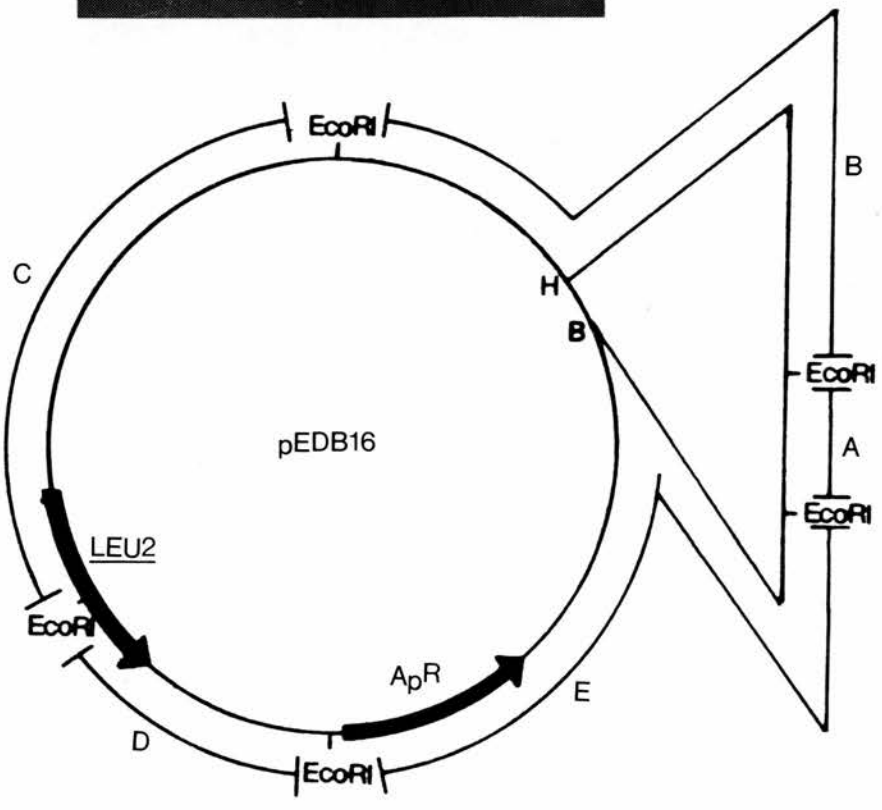
Lane 1 phage λ digested with *Eco*RI and *Hind*III

Lane 2 pEDB16 digested with *Eco*RI

Lane 3 pTN64 digested with *Eco*RI.

Cleavage of pEDB16 with *Eco*RI releases five fragments of approximately 4.6, 4.2, 2.5, 2.2 and 1.2kb, four of these fragments are also seen when pTN64 is cleaved with *Eco*RI, but the 2.5kb fragment is replaced by a larger fragment of approximately 6kb; the Tn5 molecule present in pTN64 is therefore inserted into the 2.5kb *Eco*RI fragment. Diagram B is a map of pEDB16 with the *Eco*RI restriction sites and the size of the predicted fragments marked; only Tn5 insertions into the 1.2kb and 2.5kb *Eco*RI fragments (fragments A and B respectively) were taken for further analysis.





Fragment A - 1.2 kb
B - 2.5 kb
C - 4.2 kb
D - 2.2 kb
E - 4.6 kb

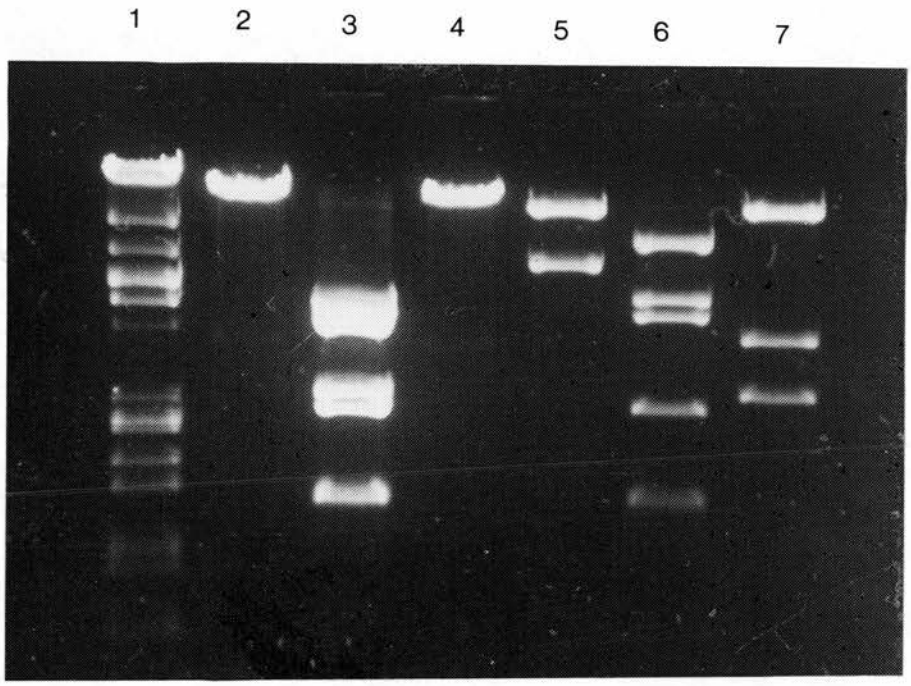
Figure 4.3 ***Bam*HI and *Hind*III digests to accurately map the position of Tn5 insertions.**

A. Restriction digests of pEDB16 (14.6kb) and pTN64 (20.3kb).

Lane1	λ *Eco*RI, *Hind*III
Lane 2	pEDB16 *Bam*HI
Lane 3	pEDB16 *Eco*RI
Lane 4	pEDB16 *Hind*III
Lane 5	pTN64 *Bam*HI
Lane 6	pTN64 *Eco*RI
Lane 7	pTN64 *Hind*III

B. An *Eco*RI digest of pTN64 (lane 6, and as in fig 4.2) confirms that the Tn5 molecule has inserted into the 2.5kb *Eco*RI fragment. *Bam*HI and *Hind*III digests were used to determine the exact position of insertion; pEDB16 contains only a single site for each of these enzymes (lanes 1 and 3 respectively). Cleavage of pTN64 with *Bam*HI produces two fragments: a large fragment that cannot be sized accurately, but is deduced to be 14.6kb, since the smaller fragment is 5.7kb. The 5.7kb fragment (fragment B) contains 2.6kb of Tn5 DNA, indicating that the transposon must have inserted 3.1kb away from the *Bam*HI site of pEDB16. This conclusion is confirmed by the *Hind*III digest, which releases three fragments: a large fragment deduced to be 14.6kb, and two smaller fragments of 3.3kb and 2.4kb; the 3.3kb fragment is an internal Tn5 fragment. The 2.4kb fragment (fragment A) contains 1.2kb of Tn5 DNA, indicating that the transposon inserted 1.2kb away from the *Hind*III site of pEDB16. The position of insertion deduced from the *Bam*HI and *Hind*III digests are identical. B (*Bam*HI), H (*Hind*III).

A



B

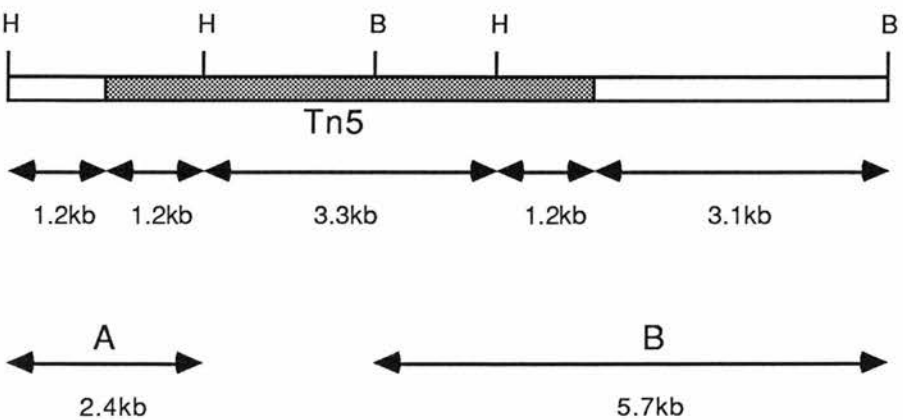


Figure 4.4 **Ability of Tn5 mutagenised pEDB16 plasmids to complement the *sec1-1* mutation.**

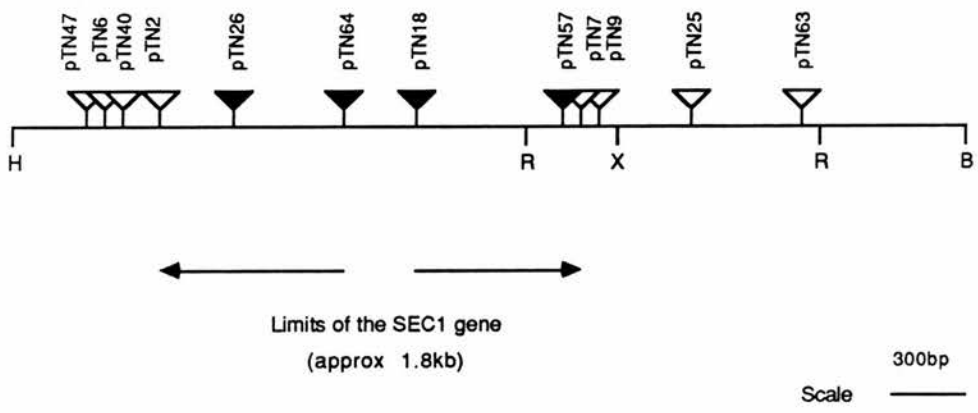
A. Each pTN plasmid was introduced into ABY12 and then tested for the ability to complement the *sec1-1* mutation at 37°C as described in the text. Complementation is denoted as (+), and failure to complement as (-).

B. Shows the position of the twelve different Tn5 insertions analysed, and their effects on the *sec1-1* complementing activity of pEDB16. Those insertions that had no effect on the ability of pEDB16 to complement the *sec1-1* mutation are shown as ▽, whereas those insertion that abolished *sec1-1* complementing activity are shown as ▼. Some restriction sites are shown B (*Bam*HI), H (*Hind*III),R (*Eco*RI), X(*Xho*I).

A

PLASMID	Number of transformants tested	Ability to grow at 37°C
pTN2	10	+
pTN6	9	+
pTN7	10	+
pTN9	10	+
pTN18	10	-
pTN25	10	+
pTN26	10	7+ 3-
pTN40	10	+
pTN47	5	+
pTN57	10	-
pTN63	10	+
pTN64	10	-

B



Chapter Five.

Gene disruption: proof that the cloned fragment of yeast genomic DNA carries the authentic *SEC1* gene.

5.1 Introduction.

In chapter three I demonstrated that the gene which has been isolated from the Nasmyth library can complement the *sec1-1* mutation when it is integrated into the genome at single copy. This is strong circumstantial evidence that the gene is the authentic *SEC1* gene, but formal genetic proof of this point is still required. A gene disruption experiment proves that a cloned fragment carries the gene in question (Rotstein 1983). The results described in chapters three and four provide all the information that is required to undertake such an experiment: the gene has been accurately mapped by Tn5 mutagenesis and has been found to span a region containing some useful restriction sites. In this chapter I describe disruption of the *SEC1* gene, by insertion of the *URA3* gene, and demonstrate that this construct integrates into the chromosome at the *SEC1* locus.

5.2 Insertion of the *URA3* gene into the *SEC1* gene and disruption of the chromosomal *SEC1* gene.

A 1.1kb *Hind*III fragment, carrying the *URA3* gene, was taken from YEp24 (Botstein *et al.,* 1979) and ligated into the *Hind*III site of pK19 - a derivative of pUC19 that confers Km^R instead of Ap^R (Pridmore 1987). A recombinant plasmid was isolated that actually contains two 1.1kb *Hind*III fragments, and has been termed pK19URA3 (see fig 5.1). The *URA3* gene was excised from pK19URA3 on a 1.1kb *Sma*I fragment and ligated into the *Hinc*II site of pMEΔ0, which by restriction mapping and transposon mutagenesis is deduced to be in the *SEC1* gene, to form plasmid pSKO1.

It was thought likely that the *SEC1* gene would encode an essential function, in which case disruption of the *SEC1* gene in a haploid strain would be a lethal event; all experiments were therefore undertaken using a diploid strain. Approximately 10μg pSKO1 was digested with *Cla*I and then used to transform MEY121 (*ura3-52/ura3-52*, *SEC1/sec1-1* diploid) to uracil prototrophy. The *Cla*I

fragment which carries the *URA3* gene (see fig 5.2) has *SEC1* sequences at both its termini; integration will therefore occur either at the wild type *SEC1* locus or the *sec1-1* mutant allele. This will give rise to temperature sensitive and temperature resistant diploid transformant strains respectively, at approximately equal frequencies. If integration were to occur at some other undefined locus, all transformants would be temperature resistant. Transformants were selected by growth on minimal medium in the absence of uracil, at 26°C: fifty of these transformants were patched onto a minimal medium, incubated at 26°C for 24hr, and then replica plated onto complete medium and incubated at either 26°C or 37°C. Eighteen transformants failed to grow at 37°C consistent with integration at the *SEC1* locus.

Integration has also been investigated by Southern hybridisation. Genomic DNA was prepared from the diploid strain MEY121, from the parents of this strain (MEY1 and MEY12), and from five URA^+ transformants (MEY121K1-MEY121K5) that were either temperature sensitive (MEY121K2, K4, and K5) or temperature resistant (MEY121K1 and K3). DNA was cleaved with *Hind*III, separated by gel electrophoresis and transferred to nitrocellulose, and then probed with pHX18 (see fig 5.3). The diploid strain, and its parents, contain only a single fragment, of 4.6kb, that hybridises with this probe; this fragment will be present in two copies in the diploid strain. The URA^+ transformants however, each contain three fragments: a fragment of 4.6kb representing the non-disrupted *SEC1* allele, and fragments of 3.4kb and 2.5kb produced by insertion of the *URA3* gene into the other *SEC1* allele. It is important to note that disrupted temperature resistant and temperature sensitive diploid strains give exactly the same band pattern in this experiment, confirming that integration has occured at one locus. It is also of interest that in all five URA^+ strains only one chromosome has experienced integration, suggesting that disruption of both *SEC1* alleles in MEY121 is a lethal event. This question is further investigated in section 5.3.

5.3 Sporulation and tetrad dissection of a temperature resistant and a temperature sensitive URA$^+$ diploid strain.

The diploid strains MEY121K1 (temperature resistant) and MEY121K4 (temperature sensitive) were patched onto minimal medium, incubated at 26°C for 36hr, and then replica plated onto sporulation medium. After four days incubation at 26°C, approximately 60% of cells had undergone sporulation events. Twelve four-spored asci derived from each diploid were dissected and the spores picked onto complete medium and incubated at 26°C. Every ascus except one gave rise to two viable strains and two non-viable strains (see fig 5.4); in the case of MEY121K1 (integration into the *sec1-1* allele) the viable spores gave rise to temperature resistant clonies which were ura$^-$, whereas MEY121K4 gave rise to temperature sensitive ura$^-$ colonies. Other nutritional markers that were analysed (*his3-11*, *trp1-289*) segregated randomly (data not shown). These characteristics confirm that the cloned gene maps to the *SEC1* locus and establishes *SEC1* as a gene essential for cell viability. Microscopic examination of spores containing a disrupted *SEC1* gene revealed that they had germinated and had passed through a few rounds of cell division (data not shown). These spores presumably contained enough residual *SEC1* gene product to support germination and growth for this period.

5.4 Summary

I have used a gene disruption experiment to demonstrate conclusively that the gene which has been isolated by its ability to complement the *sec1-1* mutation is the authentic *SEC1* gene. The *URA3* gene was used to disrupt the *SEC1* gene and a linear DNA fragment, with *SEC1* coding sequences at it termini, used to transform a *SEC1/sec1-1*, *ura3-52/ura3-52* diploid strain to uracil prototrophy. Some 36% URA$^+$ transformants simultaneously became temperature sensitive. Dissection of tetrads from a temperature sensitive URA$^+$ diploid transformant produced a segregation of 2 *sec1-1,ura3-52* : non viable (inferred to be URA$^+$) strains, and

similiarily a temperature resistant URA^+ diploid transformant gave 2 *SEC1/ura3-52* : 2 non viable (inferred to be URA^+) strains; the tight linkage between the temperature sensitive/resistant and ura^- phenotypes confirms integration at the *SEC1* locus. The fact that only two spores per tetrad were viable demonstrates that the *SEC1* gene product is essential for cell viability even at 26°C, removing the possibility that it could be required only at 37°C; or that temperature sensitivity is due to an effect of a defective protein rather than absence of function.

Figure 5.1 **Formation of pK19URA3.**

Plasmids YEp24 and pK19 were cleaved with *Hind*III and the resulting fragments introduced into a ligation reaction. DNA molecules were used to transform *E. coli* NM522 to Km^R, in the presence of IPTG and X-GAL, allowing strains carrying regenerated pK19 to be distinguished from strains carrying recombinant plasmids. Recombinant plasmids were screened for those which contained the 1.1kb *Hind*III fragment of YEp24 which carries the *URA3* gene; one plasmid, termed pK19URA3, was identified that has two tandem copies of this fragment.

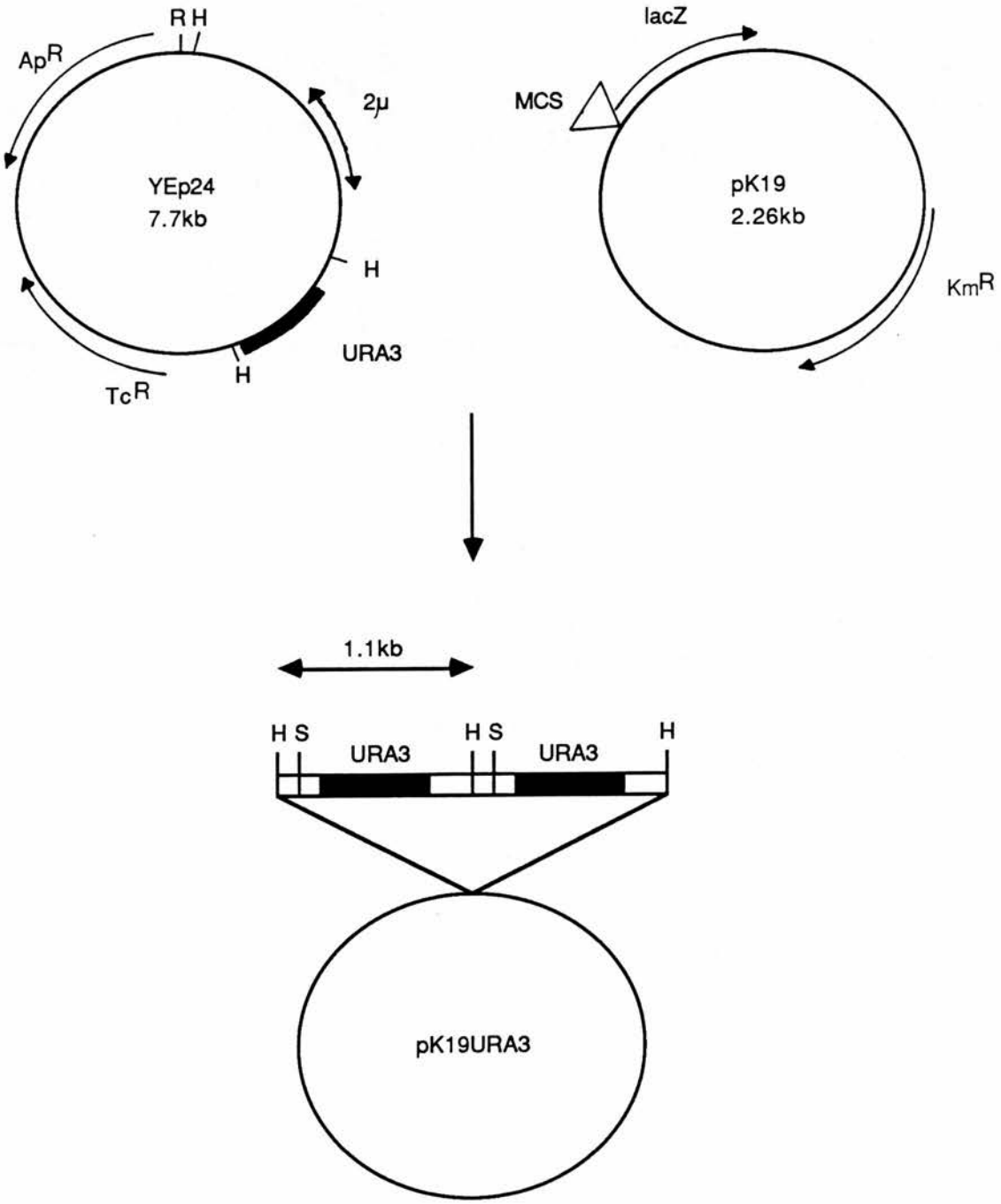
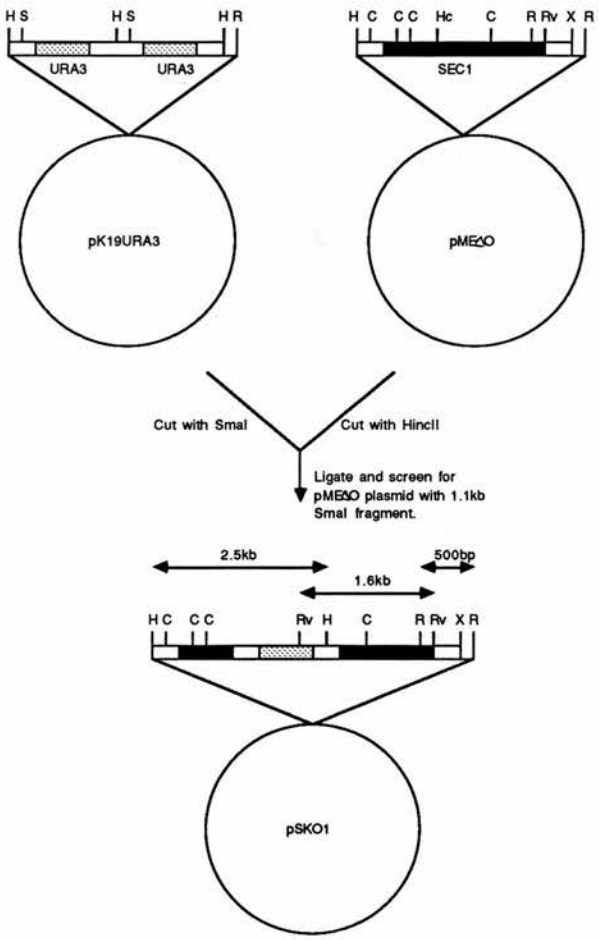

R H
Ap^R
2μ
YEp24
7.7kb
H
URA3
H
Tc^R
lacZ
MCS
pK19
2.26kb
Km^R
1.1kb
H S
URA3
H S
URA3
H
pK19URA3

Figure 5.2 **Insertion of the *URA3* gene into the *SEC1* gene.**

Plasmid pK19URA3 was cleaved with *Sma*I, releasing the *URA3* gene on a 1.1kb fragment of DNA. pK19URA3 SmaI fragments were introduced into a ligation reaction with pMEΔ0 that had been linearised with *Hinc*II. Ligated DNA was used to transform *E. coli* NM522 cells to Ap^R, which were then screened for a derivative of pMEΔ0 that had the 1.1kb *Sma*I fragment inserted into the *Hinc*II site. The photograph shows restriction digests of such a derivative, alongside the same digests of pMEΔ0 for comparison

Lane1	λ *Eco*RI, *Hind*III
Lane2	pMEΔ0 *Eco*RI
Lane3	pMEΔ0 *Eco*RV
Lane4	pMEΔ0 *Hind*III
Lane5	pSKO1 *Eco*RI
Lane6	pSKO1 *Eco*RV
Lane7	pSKO1 *Hind*III

Insertion of the 1.1kb *Sma*I fragment introduces additional *Hind*III and *Eco*RV restriction sites to produce fragments of 2.5kb and 1.6kb respectively, and increases the size of the largest fragment of pMEΔ0 in an *Eco*RI digest; these characteristics were used to identify pSKO1. Single lines represent vector sequences, and blocks represent cloned DNA fragments. Some restriction sites are shown for individual plasmids: C (*Cla*I), H(*Hind*III), Hc (*Hinc*II), R (*Eco*RI), Rv (*Eco*RV), X(*Xho*I).


H S
H S
H R
URA3
URA3
pK19URA3
H C C C Hc C R Rv X R
SEC1
pMEΔO
Cut with SmaI
Cut with HincII
Ligate and screen for pMEΔO plasmid with 1.1kb SmaI fragment.
2.5kb
500bp
1.6kb
H C C C Rv H C R Rv X R
pSKO1

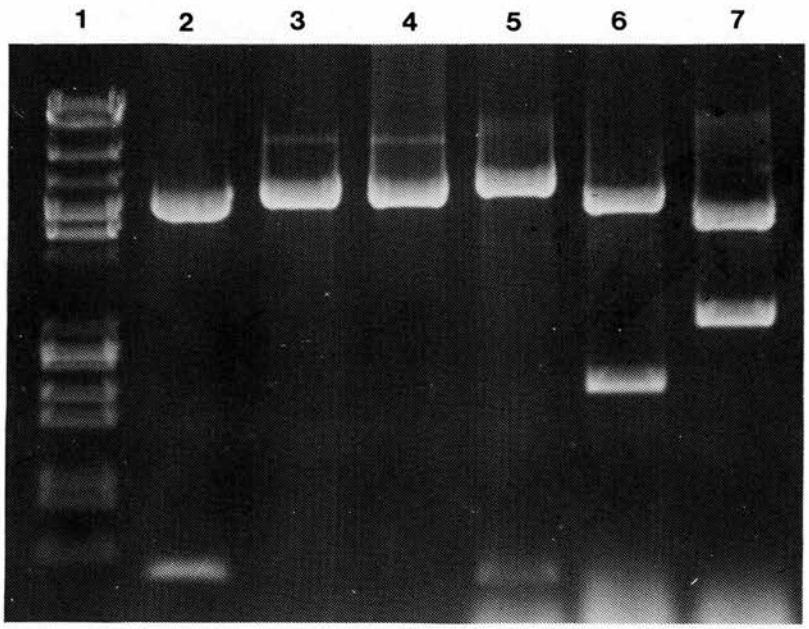

1
2
3
4
5
6
7

Figure 5.3 Disruption of the chromosomal *SEC1* gene.

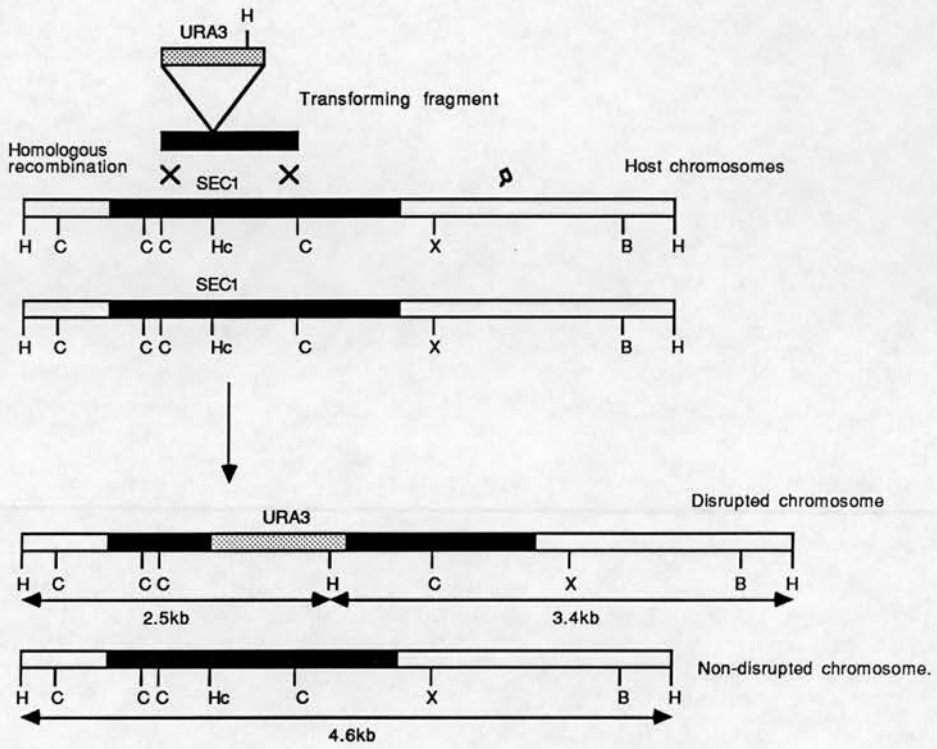
A. A schematic diagram of the integration event. Plasmid pSKO1 was cleaved with *Cla*I and then used to transform MEY121 to uracil prototrophy. The *Cla*I fragment that carries the *URA3* gene becomes established by homologous recombination into the host chromosome; integration will therefore be targetted to the *SEC1* locus. B (*Bam*HI), C (*Cla*I), H (*Hind*III), Hc (*Hinc*II), X (*Xho*I).

B. Southern hybridisation was used to confirm the nature of integration events. Genomic DNA was cleaved with *Hind*III and, after electrophoresis and transfer to nitrocellulose, was probed with pHX18.

Lane1 MEY121K1
Lane2 MEY121K2
Lane3 MEY121K3
Lane4 MEY121K4
Lane5 MEY121K5
Lane6 MEY1
Lane7 MEY12
Lane8 MEY121

The observed band patterns agree exactly with the predictions made in diagam A. In the three untransformed strains (MEY1, MEY12, and MEY121) only fragments of 4.6kb hybridise with the probe, whereas the URA$^+$ transformants have three fragments: the 4.6kb fragment as above, and fragments of 3.4kb and 2.5kb which are the result of disruption with *URA3* gene.

A


URA3
H
Transforming fragment
Homologous recombination
SEC1
Host chromosomes
H C C C Hc C X B H
SEC1
H C C C Hc C X B H
Disrupted chromosome
URA3
H C C C H C X B H
2.5kb
3.4kb
Non-disrupted chromosome.
H C C C Hc C X B H
4.6kb

B

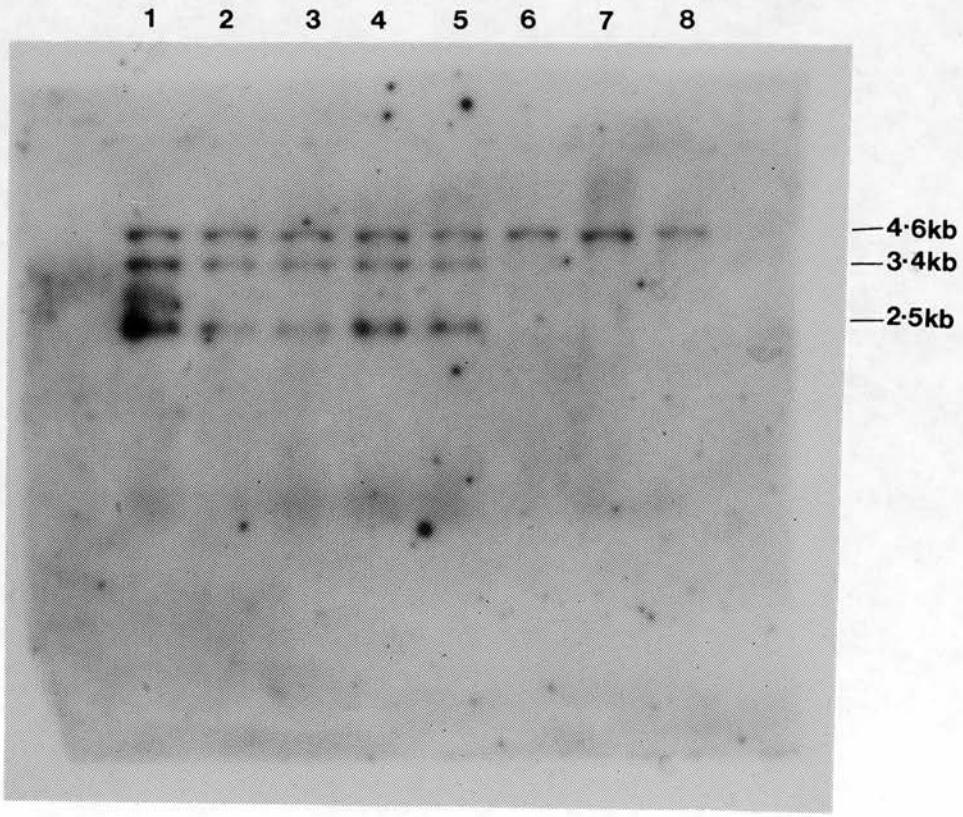
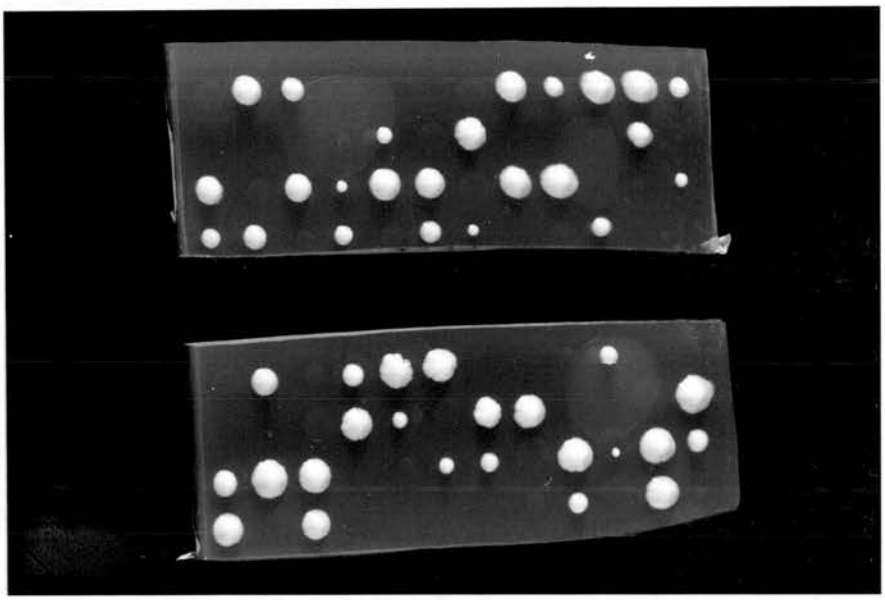

1 2 3 4 5 6 7 8
4·6kb
3·4kb
2·5kb

Figure 5.4 **Dissection of tetrads from a temperature resistant and a temperature sensitive URA^+ diploid.**

Twelve four-spored asci from a temperature resistant URA^+ diploid (top panel) and a temperature sensitive URA^+ diploid (bottom panel) were dissected. Spores were grown at 25°C on complete medium for three days and then photographed. See text for further details. Many thanks are due to Dr. Ian Dawes (Dept. Microbiol., Univ. of Edinburgh.) for carrying out the tetrad dissection.



Chapter Six.

Characterisation of the *SEC1* gene and its product: Northern hybridisation, *in vitro* transcription and translation, and nucleotide sequencing.

6.1 Introduction.

In previous chapters I have described the isolation of the *SEC1* gene by its ability to complement the *sec1-1* mutation. In this chapter I describe further studies that I have undertaken, including Northern hybridisation experiments, *in vitro* transcription and translation of *SEC1* mRNA, and nucleotide sequencing, which have further characterised the *SEC1* gene and its product (Sec1p).

6.2 The *SEC1* gene encodes a transcript of 2.5kb.

Northern hybridisation experiments have confirmed that the *SEC1* gene, identified by subcloning and transposon mutagenesis is expressed in actively growing yeast cells. Total RNA was prepared from DBY746 that had been transformed to leucine prototrophy with either pEDB16 or YEp13. RNA was separated by gel electrophoresis, transferred to a nylon membrane, and probed with the 4.3kb *Hind*III-*Bam*HI fragment. Two transcripts of approximately 2.5kb and 5.0kb were detected in RNA prepared from DBY746 transformed with pEDB16, both of which are sufficent in size to be transcribed from an open reading frame of 1.8kb (see fig. 6.1). The 2.5kb transcript was also observed in RNA prepared from DBY746 transformed with YEp13 on prolonged exposure of the filters (data not shown), and thus is a candidate for the *SEC1* mRNA.

To confirm that this transcript represents *SEC1* mRNA, and to determine the orientation of the *SEC1* gene, RNA was probed with a smaller fragment of DNA from the 4.3kb *Hind*III-*Bam*HI fragment. The 2.9kb *Hind*III-*Xho*I fragment, which houses the complete *SEC1* gene, was ligated into the *Hind*III-*Sal*I restriction sites of bacteriophages M13mp18 and mp19, to form pHX18 and pHX19 respectively (see chapter 2). Single stranded pHX18 and pHX19 DNAs were harvested and used to probe similiar blots to the one described above. Due to the opposite orientation of the cloning sites in mp18 and mp19, pHX18 and pHX19 contain opposite strands of the

*Hind*III-*Xho*I fragment, ie either the *SEC1* coding or non-coding strand, but only the coding strand will hybridise to the mRNA. A single transcript is detected using pHX18 as a probe (see fig. 6.2); this is the same size as the major transcript, shown in fig. 6.1, detected when the 4.3kb *Hind*III-*Bam*HI fragment was used as a probe. It is most likely therefore that this transcript is derived from the *SEC1* gene. Conversely, no transcript was detected using pHX19 as a probe, suggesting that pHX19 contains the non-coding strand of the *SEC1* gene. This result therefore establishes the orientation of the *SEC1* gene in the *Hind*III-*Xho*I fragment: the 5' end of the gene is towards the *Hind*III restriction site and the 3' end towards the *Xho*I restriction site, as depicted in fig. 6.2. The origin of the larger transcript shown in fig. 6.1 is unknown; but it is probably due to spurious initiation of transcription in YEp13 sequences.

6.3 Coupled *in vitro* transcription-translation of the *SEC1* gene.

The potential for the *SEC1* gene to encode a polypeptide was tested by *in vitro* transcription-translation using a prokaryotic cell free system (De Vries and Zubay 1967, Zubay 1973). This system contains a crude preparation from *E. coli* which contains all the necessary enzymes and factors required for transcription and translation, which is supplemented with amino acids, an energy regenerating system and certain co-factors; all that is required to program the reaction is plasmid DNA. Newly synthesised proteins are radiolabelled by incorporation of [^{35}S]- methionine, and then visualised by SDS-PAGE with subsequent fluorography.

Eukaryotic genes can be expressed in this system provided they posses prokaryotic expression signals and do not contain an intron(s), although the difference in codon useage between prokaryotes and eukaryotes may lead to spurious products being synthesised due to premature termination of translation etc. Despite these potential problems however, yeast myosin (a protein of approximate molecular weight 200K) has been expressed from its gene (*MYO1*) in the Zubay system (Eli Orr, personal

communication).

Figure 6.3 shows the polypeptide profiles obtained when numerous plasmids were introduced into a Zubay system. All the plasmids analysed in this experiment were derivatives of YEp13 which when used to program transcription and translation directs expression of three predominant polypeptides of molecular weight 55K, 44K, and 30K, and many other minor species which are probably spurious. The larger polypeptide of approximate molecular weight 55K is even produced in a mock reaction containing no DNA (fig. 6.3, lane 6), and must therefore be expressed from contaminating DNA. The polypeptides of molecular weight 44K and 30K are seen in all the lanes in fig. 6.3 (except the mock reaction, lane 6); this distribution and the size of the two proteins are consistent with them being ß-IPM dehydrogenase (product of the *LEU2* gene, Andreadis *et al.,* 1984) and ß-lactamase (product of the Ap^R gene, Bolivar *et al.,* 1977) respectively.

In addition to the three polypeptides described above, pEDB1 directs the expression of several more polypeptides of approximate molecular weight 26K, 35K, 46K, and five polypeptides of molecular weight 69-74K. It is not possible to say whether these are all derived from separate open reading frames, or if they are the result of spurious translation. Four of the polypeptides, of molecular weight 69-72K, are also produced when pEDB16 is used to program the Zubay system, and are therefore likely candidates for the *SEC1* gene product. To determine which of these polypeptides is derived from the *SEC1* ORF, two mutagenised derivatives of pEDB16, pTN18 and pTN64 (see section 4.2), were analysed. Both of these plasmids have Tn5 insertions into the *SEC1* gene, which completely abolish the ability of the plasmids to complement the *sec1-1* mutation at 37°C. No polypeptides of approximate molecular weight 69-72K are expressed from these plasmids, consistent with each polypeptide being derived from the *SEC1* gene; presumeably the largest polypeptide (72K) represents the full length product. Interestingly, two unique polypeptides of approximate molecular weight 26K and 28K are seen in the profiles of pTN18 and

pTN64 respectively. The size of these polypeptides is consistent with them being truncated *SEC1* polypeptides: pTN18 is distal to the 5' end of the gene and directs the expression of a larger protein than pTN64, which is proximal.

6.4 Determination of the nucleotide sequence of the *SEC1* gene.

To gain more information about the *SEC1* gene and the protein it encodes, I have determined the nucleotide sequence of the *SEC1* gene. The 2.9kb *Hind*III-*Xho*I fragment has been completely sequenced, on both strands, following the strategy outlined in fig. 6.4 All six possible reading frames have been analysed, but only a single open reading frame of significant length has been identified (fig. 6.5). This ORF is 1828bp long, which could encode a polypeptide of 626 residues, and is likely to be the *SEC1* coding sequence for two reasons. First, the location of the ORF corresponds to that region defined by transposon mutagenesis to be essential for complementation of the *sec1-1* mutation; and second, the ORF is in the same orientation to that determined by the Northern hybridisation experiments described above (section 6.2).

6.4A Analysis of the *SEC1* open reading frame.

The ORF identified by nucleotide sequencing could encode a polypeptide of 626 amino acids. This polypeptide (Sec1p) has a predicted molecular weight of 72 936, consistent with the size of the candidate Sec1p seen in the *in vitro* transcription - translation experiment.

The amino-terminal residues of Sec1p do not bear any resemblance to a typical amino-terminal signal sequence: this region contains only nine hydrophobic amino acids in the first twenty residues (scoring Ala, Cys, Leu, Ile, Met, Val, Trp, and Phe as hydrophobic residues). Furthermore, analysis of the hydropathy of Sec1p using the method of Kyte and Doolittle (1982) reveals that there are no candidate internal

hydrophobic domains that could direct translocation into a membrane (data not shown). Two other proteins thought to play a role in secretion, Ypt1p and Sec4p, are peripherally bound to membranes (see sections 1.4.1 and 1.4.2). It has been demonstrated for Ypt1p that attachment is accomplished by palmityolation of cysteine residues in the sequence GlyGlyCysCys, which forms the carboxy-terminus of the protein. In contrast to Ypt1p, the carboxy-terminus of Sec1p is AlaThrArgTyr, suggesting that it is not a substrate for palmityolation; therefore in all probability Sec1p will be found in the cytosolic fraction of the cell.

The second codon of Sec1p is UCU, encoding a serine residue. Bachmair *et al.,* (1986) have demonstrated that in yeast, the second position of *E. coli* ß-galactosidase has a great effect on the stability of the protein; they further infer that this is likely to be true for all unblocked non-compartmentalised proteins. Serine, along with methionine, alanine, glycine, threonine and valine have been categorised as stabilising amino acid residues, and are found to occupy the second position in a protein in 63% of all yeast proteins (Bachmair *et al.,* 1986). This could imply that Sec1p is a particularly stable protein; consistent with this, is the observation that haploid strains in which the *SEC1* gene has been disrupted can germinate and undergo one to three rounds of cell division. Alternatively, the presence of UCU in the second codon position may effect translation of mRNA. This has been demonstrated for *E. coli* mRNA, where UCU is the second most frequently used codon at this position. A mutational analysis of the second codon in the *lacZ* mRNA demonstrated that the nature of the second codon affects expression over a twenty fold range (Looman *et al.*, 1987).

6.4B Codon useage in the *SEC1* gene.

Genes that are highly expressed in *S.cerevisiae* show a distinct preference for the possible coding triplets, in extreme cases only 22-26 of the possible 61 constitute 93% of all the triplets. Preferred codons are thought to reflect the major species of

tRNA in *S. cerevisiae*. The codon bias index (CBI) is a measure of the fraction of codon choices which is biased to 22 preferred triplets, a value of one indicates that for all of the triplets in the mRNA only preferred codons are used, whereas a value of zero reflects total random choice. Using the method of Bennetzen and Hall (1982a) I have determined the CBI of the *SEC1* gene to be 0.305. Highly expressed genes, such as those encoding glyceraldehyde-3-phosphate dehydrogenase and alcohol dehydrogenase isozyme 1 have reported CBI values greater than 0.9; whereas the iso-2 cytochrome c gene, which is expressed at low levels, has a CBI value of 0.15 (Bennetzen and Hall 1982a). In comparison therefore, a CBI value of 0.305 predicts that the *SEC1* gene will only be expressed at moderately low levels.

6.4C Analysis of the non-coding 5' and 3' flanking regions.

Specific sequence motifs exist in the 5' untranslated region of eukaryotic genes that are thought to play a significant role in initiation and regulation of mRNA transcription. The TATA element (consensus sequence 5'TATAA3') is an essential component of many promoters; its function is not completely understood but it is believed to to be involved in positioning of RNA polymerase II transcription start sites (for review see Breathneach and Chambon 1985). In higher eukaryotes the TATA sequence is almost invariably located 25-30bp upstream from the mRNA initiation site (Gannon *et al.*, 1979), but in yeast it can be found up to 150bp upstream of the ATG codon, and many promoters contain more than one TATA element (Hahn *et al.*, 1985). In contrast, the sequence 5'TATAA3' is not found in the *SEC1* promoter, but the sequences 5'TGTAA3' and 5'AATAA3' which resemble the consensus are located at -73 to -69 and -59 to -55 respectively, although it has not been determined if these are functional. The absence of a consensus TATA element is consistent with the hypothesis that the *SEC1* gene is expressed at low levels. The *CDC37* and *CDC39* are two other examples of genes that do not have a TATA element in their promoter and are also expressed at low levels: both genes encode approximately five mRNA molecules per haploid cell and have codon bias index values of below 0.15

(Ferguson *et al.,* 1986).

Dobson *et al.,* (1982) have reported a correlation between highly expressed genes of *S. cerevisiae* and a pyrimidine rich region, in 5' flanking sequences, followed ten nucleotides downstream by the sequence 5' CAAG 3'; initiation of transcription is thought to occur in the latter sequence (Kingsman *et al.,* 1985). A block of pyrimidine residues is found in the *SEC1* promoter between -132 and -119 but this does not precede the tetranucleotide sequence 5' CAAG 3'; between nucleotide position -119 and the ATG codon however, the related sequence 5' TAAG 3' (PyAAG) occurs three times. One of these three sequences could provide the start site for the *SEC1* transcript, although this is yet to be determined.

Numerous sequences have been associated with transcriptional termination in *S. cerevisiae*, but a general consensus sequence has yet to be recognised. In several genes, termination occurs in the sequence 5' TAAATAAG 3' (Bennetzen and Hall 1982b); no example of this sequence is found in the 3' flanking regions of the *SEC1* gene. Alternatively, termination in the *CYC1* gene is dependent upon the sequence 5'TAG...TAGT...(A/T rich)TTT3' which has since been found in a similiar position in other yeast genes (Zaret and Sherman 1982). Again, no example of this sequence can be found downstream of the *SEC1* termination codon; termination of transcription, therefore either occurs at these sequences downstream of the *Xho*I site (this sequence has not been determined), or at some other undefined sequence.

6.4D Context of the intiation codon.

In animal cells it is generally accepted that after the 40S ribosomal subunit has bound to the 5' Cap-site, the subunit scans the leader until the first AUG codon is encountered, at which point translation initiation occurs (Kozak 1981). The context of the AUG, however, acts as an important signal to trigger initiation events. Hamilton *et al.,* (1987) compared the DNA sequences around the ATG start codon of

96 genes from *S. cerevisiae*; they found that 81% of these ATG codons were preceded by an A residue at position -3, and 61% by an A residue at -1: the *SEC1* ATG is preceded by an A residue at position -3 but by a G residue at -1. In those genes studied by Hamilton *et al.*, (1987) only 9% were found to have a G residue at position -1; in fact, G residues were rare throughout the leader, especially in the five positions immediately preceding the ATG codon. The *SEC1* transcript however, has eight G residues between -1 and -30, two of which occur between -1 and -5.

A strong bias also exists for the first three bases downstream of the start codon. The +4 and +6 positions are occupied by a T residue in 38% and 57% of those genes studied by Hamilton *et al.*, (1987) respectively; and a C residue is found at +5 in 52% of the cases. Thus, the first codon after the initiating ATG is usually TCT; this is true of the *SEC1* gene. In summary, the studies of Hamilton *et al.*, (1987) reveal the consensus DNA sequence 5' A/TAA/CAA/CA ATGTCT/C 3', whereas the equivalent sequence in the *SEC1* transcript is 5' GGAACGATGTCT 3', reinforcing the hypothesis that the *SEC1* gene will be poorly expressed.

The ATG which forms the start of the *SEC1* ORF is preceded by an out of phase ATG at -20, which is followed by an ORF of only ten codons. This ATG, however, is not found in a favourable environment: the -3, -1, and +4 positions are occupied by C residues, only the +6 position, which is a U, is favourable. It is therefore uncertain whether this ATG would act as initiating signal for some fraction of the ribosomes scanning the *SEC1* mRNA molecule.

6.4E Analysis of potential splicing sites.

Splicing of mRNA molecules in *S.cerevisiae* is rare, only nine genes have been identified that are synthesised as pre-mRNA, and subsequently matured by removal of an intron. As in higher eukaryotes there are stringent sequence requirements at

the 5' and 3' exon/intron boundaries (for review see Breathneach and Chambon 1981). The 5' donor site has been defined by the consensus sequence 5'GTATGT3' and the 3' acceptor site by the sequence 5'C/UAG3'. In *S. cerevisiae* however, there is also an absolute requirement for the sequence 5'TACTAAC3' which is found 16-64 nucleotides upstream of the 3' acceptor site (Langford and Gallwitz 1983). Analysis of the *SEC1* nucleotide sequence revealed the presence of possible splicing sequences within the large ORF described above: the sequence 5'TACTAAC3' is found at 495-501, which is preceded by a candidate 5' donor site, sequence 5'GTATCG3', at 251-256; and followed by numerous 3' acceptor sites between 504-530.

To determine if the *SEC1* transcript is spliced I have analysed mRNA prepared from a wild type strain and a mutant strain that is defective in splicing. The *rna2* mutation is temperature sensitive, such that at 37°C, *rna2* mutants accumulate unspliced transcripts at the expense of mature mRNA molecules (Hartwell 1967, Roshbash *et al.*, 1981, Fried *et al.*, 1981). Unspliced and mature mRNA molecules can be distinguished by Northern hybridisation, since removal of an intron leads to a reduction in the size of the mRNA. A nylon membrane, onto which RNA extracted from a wild type strain and an *rna2* mutant strain had been blotted, was the kind gift of Drs. Derek Jamieson and Jean Beggs (Dept. Mol. Biol., Univ. of Edinburgh). This membrane was probed with plasmid pMEΔ0 (see chapter 2) that had been linearised by cleavage with *Hind*III. The results are shown in fig. 6.6: in the wild type strain, at both 26°C and 37°C, a single transcript of approximately 2.5kb was detected; the same transcript is also detected in RNA extracted from the *rna2* mutant at 26°C. No difference however, can be detected between the size of this transcript and that detected in an *rna2* mutant at 37°C; therefore either the *SEC1* mRNA is not subject to splicing, or the number of spliced mRNA molecules is so low they cannot be detected in this experiment.

6.4F Comparison of the *SEC1* gene product with proteins in the NBRF database.

The polypeptide sequence of Sec1p was compared to entries in the NBRF database (version 16), using the Lipmann and Pearson FASTP program in KTUP1 mode (Lipman and Pearson 1985). Of the proteins that showed some similarity to Sec1p, the most significant was the product of the *Schizosaccharomyces pombe nim1*$^{+}$ gene. This gene was identified by its ability, when cloned on a multi-copy plasmid, to suppress a *cdc25*^{ts} mutation, and has therefore been implicated in the control of cell division (Russell and Nurse 1987). The nucleotide sequence of *nim1*$^{+}$ reveals that it could encode a polypeptide of 370 residues (Nim1p), of molecular weight 50 000, that could be a protein kinase since it was found to be 20-35% identical to the catalytic domains of a family of protein kinases, including cAMP-dependent protein kinase and the v-src oncogene product. The similarities are concentrated in the regions of the ATP binding site, and in the region where it is known that in certain protein kinases a tyrosine or threonine residue is phosphorylated.

Figure 6.7 shows a comparison of Sec1p and (Nim1p) protein: in this configuration the two proteins show 25% identity over a region of 136 amino acids, but this similarity does not suggest Sec1p to be a protein kinase, since none of the residues that are absolutely conserved in twelve protein kinase sequences (Hunter and Cooper 1985) are represented in Sec1p.

6.5 Summary.

In this chapter I have described Northern hybridisation experiments that have enabled *SEC1* mRNA to be detected in wild type cells. This transcript is approximately 2.5kb long, consistent with the size of the *SEC1* gene as defined by transposon mutagenesis. Using strand specific probes I have also demonstrated that the *SEC1* gene is orientated with its 5' terminus proximal to the *Hind*III site, and

runs 5' to 3' towards the *Xho*I site. To confirm that the *SEC1* gene has the potential to encode a polypeptide, I have expressed the gene using a prokaryotic *in vitro* coupled transcription and translation system. A polypeptide of approximate molecular weight 72K is expressed from pEDB16 in this system, but is not detected when two derivatives of this plasmid (pTN18 and pTN64), containing Tn5 insertions into the *SEC1* gene, are analysed.

To gain more information about the *SEC1* gene and its product (Sec1p), I have determined the nucleotide sequence of the 2.9kb *Hind*III-*Xho*I fragment. Only a single ORF has been identified which, by the following criteria, is likely to be the *SEC1* coding sequence: it is the only ORF that begins with an ATG codon and continues for a significant length; it is in the correct orientation as elucidated by Northern hybridisation; and the ORF is located in the same region identified by transposon mutagenesis to be essential for *sec1-1* complementing activity. The *SEC1* ORF is 1828bp long, and could encode a polypeptide of molecular weight 72 936; this is consistent with the size of Sec1p observed in the prokaryotic coupled *in vitro* transcription-translation system. Two characteristics of the *SEC1* gene suggest that it is expressed at low levels only: analysis of 5' flanking sequences reveal that the *SEC1* promoter has very few of the sequence motifs that correlate with high levels of gene expression; and the codon bias index for the *SEC1* ORF is 0.305, indicating that there is no tendency for preferred codons to be used. The amino acid sequence of Sec1p has been used to search the NBRF database to discover if it bears any resemblance to other proteins of known function. Using the Lipman and Pearson FASTP program, Sec1p was found to be similiar to the *nim1*$^{+}$ protein kinase of *Schizosaccharomyces pombe*, although this resemblance does not imply Sec1p to be a protein kinase. The possible implications of this similarity will be discussed in chapter 9.

Figure 6.1 **Northern hybridisation to detect the *SEC1* transcript.**

Total RNA was extracted from DBY746 that had been transformed to leucine prototrophy with either pEDB16 or YEp13. Approximately 15µg of RNA was separated by gel electrophoresis and then blotted onto Genescreen hybridisation membrane. This filter was then probed with the 4.3kb *Hind*III-*Bam*HI fragment using stringent hybridisation conditions.

Lane1. RNA from DBY746 transformed with pEDB16

Lane 2. RNA from DBY746 transformed with YEp13.

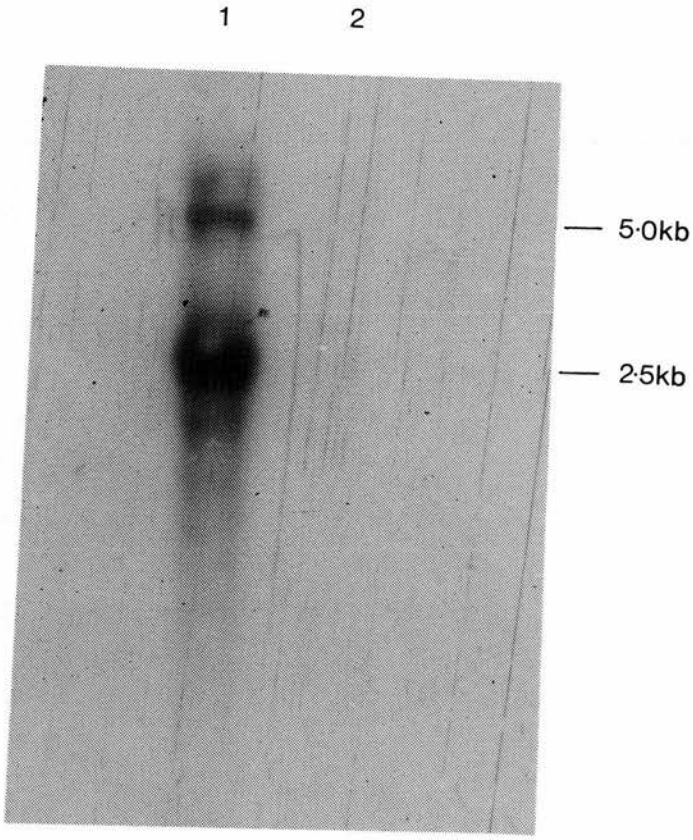

1
2
5·0kb
2·5kb

Figure 6.2 Northern hybridisation experiments using strand specific probes to detect the *SEC1* transcript and determine the orientation of the *SEC1* gene.

Single stranded pHX18 and pHX19 DNAs were prepared (see chapter 2) and used to probe blots of RNA prepared from DBY746 transformed with pEDB16.

Panel A probe: pHX18 DNA.

Panel B probe: pHX19 DNA.

A transcript was detected only when pHX18 DNA was used as a probe. This clone must therefore contain the coding strand of the *SEC1* gene, enabling the orientation of the *SEC1* gene to be drawn as in the bottom diagram.

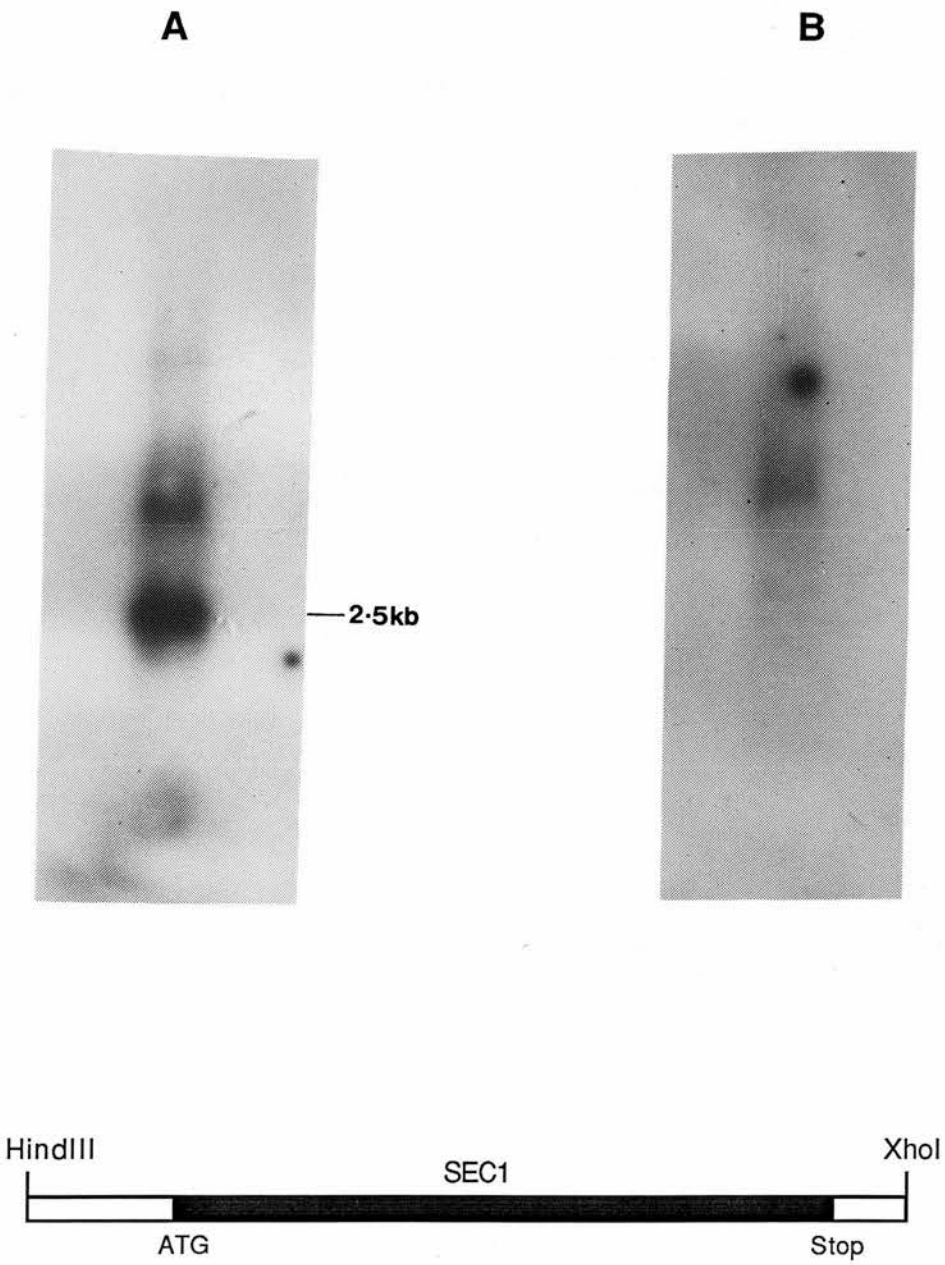

A
B
2·5kb
HindIII
XhoI
SEC1
ATG
Stop

Figure 6.3 **Coupled *in vitro* transcription and translation of the *SEC1* gene using a prokaryotic expression system.**

Plasmids that were to be analysed in the Zubay system were first purified from caesium chloride gradients. Approximately 5μg of each plasmid was mixed with the constituents of the Zubay reaction. During a 1hr incubation at 37°C newly synthesised proteins were radiolabelled by incorporation of [^{35}S]- methionine and visualised by SDS-PAGE (using a 10% w/v polyacrylamide gel) with subsequent fluorography.

Lane1 Standard molecular weight markers.

Lane2 YEp13

Lane3 pEDB1

Lane4 pEDB16

Lane5 pTN18

Lane6 pTN64

Lane7 No DNA

Standard proteins: Myosin (200K), Phosphorylase b (92.5K), BSA (69K), Ovalbumin (46K), Carbonic anhydrase (30K), Lysozyme (14.3K).

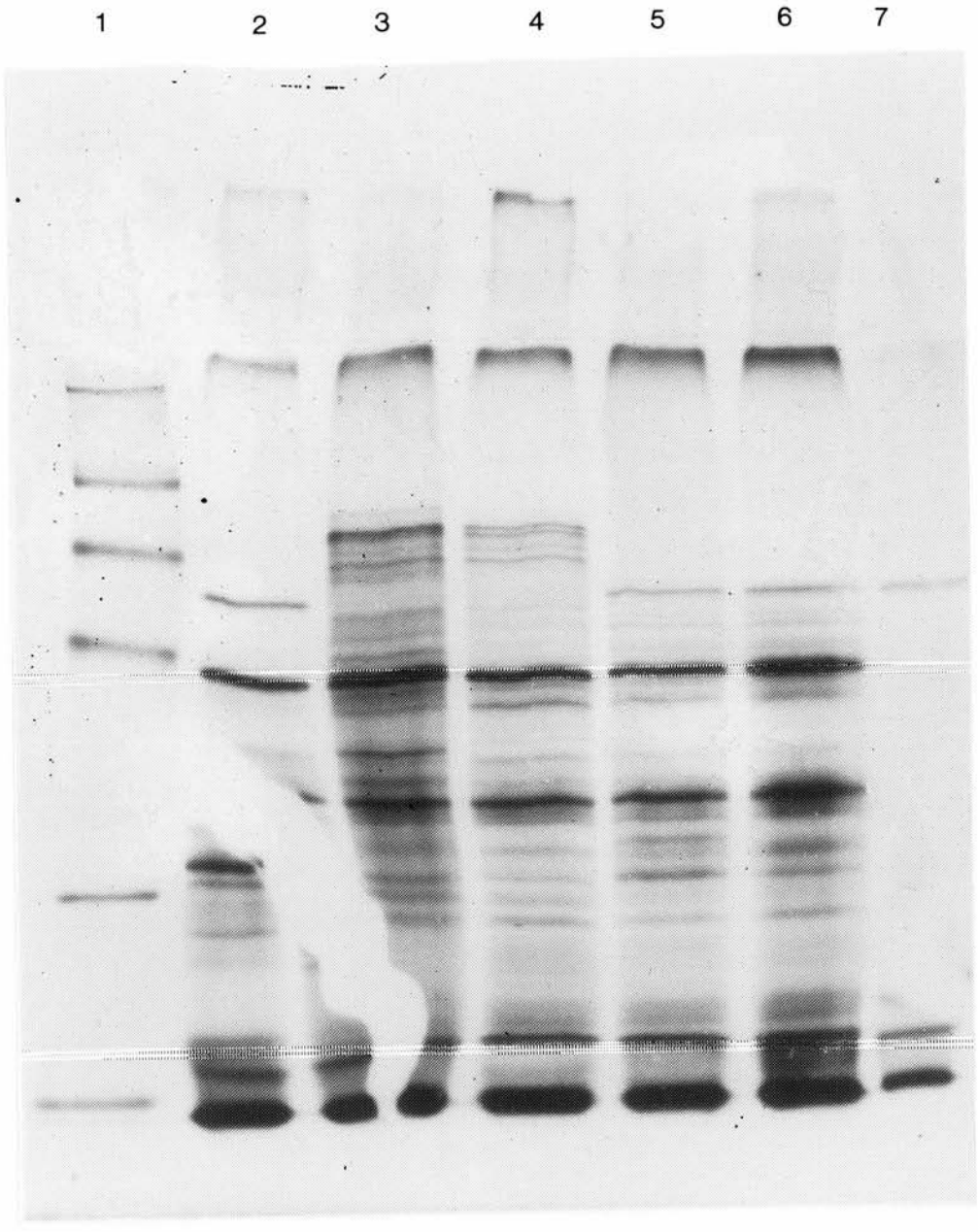

1
2
3
4
5
6
7

Figure 6.4 Sequencing strategy.

The arrows indicate the direction and extent of sequence determination. Template DNA consisted of either defined restriction fragments or *Bal*31 generated deletions cloned into the polylinker of M13mp19 (⟶); or synthetic oligonucleotides were used to prime DNA synthesis with either pHX18 or pHX19 (⟵). The *SEC*1 coding sequence is marked by the large arrow at the top of the diagram. H (*Hind*III), C (*Cla*I), R (*Eco*RI), Rv (*Eco*RV), X (*Xho*I).

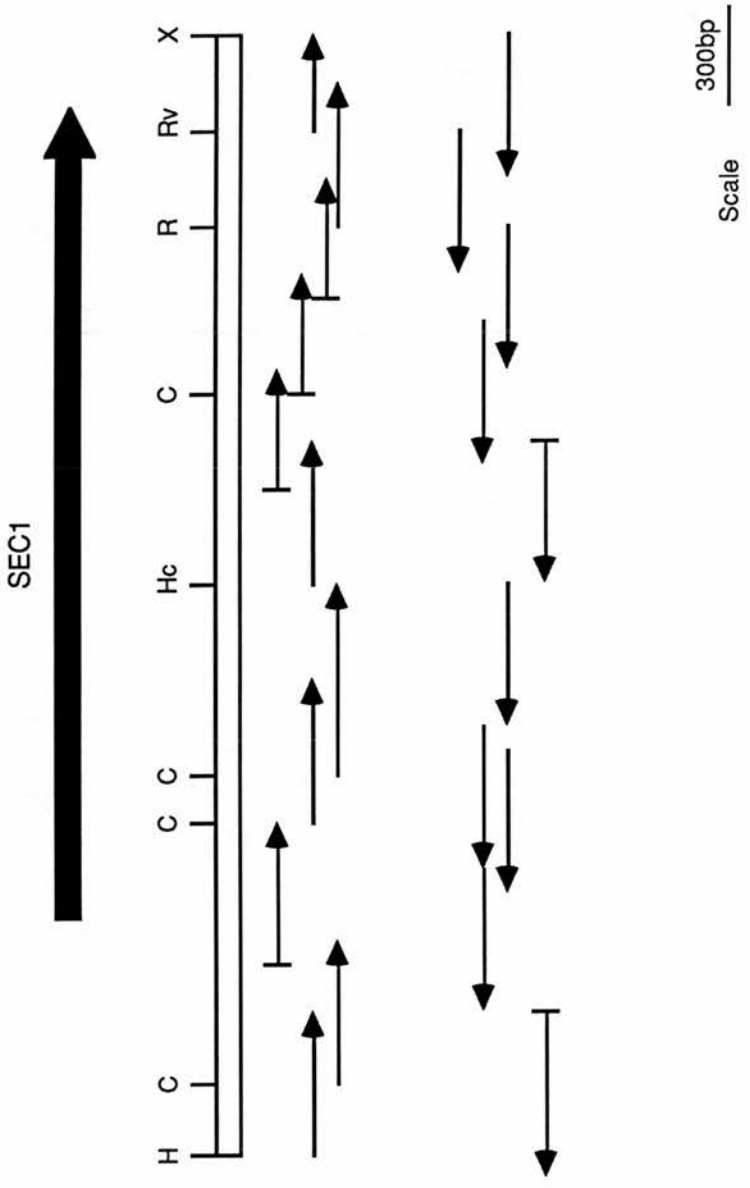

SEC1
H
C
C
C
Hc
C
R
Rv
X
Scale
300bp

Figure 6.5 Nucleotide sequence of the *SEC1* gene.

The complete sequence of the *SEC1* gene is shown as well as 5' and 3' flanking sequences. The sequence is only shown for the coding strand; the single letter amino acid code is shown below the DNA sequence. The nucleotides underlined in the 5' flanking region resemble some sequence motifs found in yeast promoters, whereas those nucleotides underlined in the open reading frame represent potential splice sites. See text for further discussion.

HindIII

-739 AAGCTTAAGAATAACAATTCTTAATGATGGCGAAAACTAATGAGCCATCACGAGAGGTGA -660
-679 GTAAATTCTGCAAAGGATGAATGACGCTCATTGTAAAACACGCGGACTGCTTCAAATTGA -600
-619 CAAACCTTGCTATACTTTAGCTGCTAATATATCATCAATACGCCTTTACTAGTAAAAAAT -540
-559 TTTTTAATCTCATCGATTTTTTTACCTCGCCATTTTTTTCGCTGATTAAAAGAAATGTAT -480
-499 CTGAGATAAGTGCCATATTTAATGACAGCAGCGTAAAATAAGAATATGTAACGATACAAT -420
-439 AAGAAAACTATGTTTATGTGTCACCTCTTGCTTGCGTCTTGGGAAAAGCATTCCCAATAA -380
-379 AATACTAAACCGGATGTAGTAGCCCTCTTAGTTTTTCTATCTAACATGTCCATCCTCCTG -320
-319 GATCAAAATATCAGTAATCTTGTATTCCTTACTTGCGATACAGCTATTATTACAGGCACT -260
-259 CTTTAAAAATCATCGAATAGCCGATCGACTACACACTTATCCCTATAACATGTGTAAGTG -200
-199 ATAGAGAGGTGAATGCGCGTCGTCTTATGTTCTCGCAATTATTGGTAGATGGTCACACAG -140
-139 CACGGTGTTTTTCTTTTGCTTAAACGATCAGTTTAAGTCCCTATTGAAACTAGTAAGACA -80
-79 TTGCTTTGTAAGACCTTGAAAATAATTAGAATCCACTGACAAAGTAGAAGAGTGTGCACA -20

-19 TGCTTGGTTTGGCGGAACG ATGTCTGATTTAATTGAATTACAGAGGAACTATTTGAT +38
M S D L I E L Q R N Y L I

+39 AGGGGTTTTGAATCAGATTGAGACGAAAAATAACTTGAAGTTTCTTATTATCGATAAAAC +98
G V L N Q I E T K N N L K F L I I D K T

+99 TGTGGAGACAATATTGAGTTATCTTTTCCTCACACCCCAAGAACTTTTAAATAATGTCAC +158
V E T I L S Y L F L T P Q E L L N N V T

+159 ATCTGTTGATTTGATTGATTCCCCAACAAGAAAAGGACAATCCTCCATCGAGGCAATTTA +218
S V D L I D S P T R K G Q S S I E A I Y

+219 CATATTGGAACCAACGAAATATAACATCAATTGTATCGATGCTGACTTCATGGTAAGACC +278
I L E P T K Y N I N C I D A D F M V R P

+279 ACCTAAGTACAGAAGATGTCACATCAGATTTTTACCAGGATTAACAAACCCTATATTTCA +338
P K Y R R C H I R F L P G L T N P I F Q

+339 GTTTTTTCAATCAAAACGTTACATTGCTCAAAATTTGGAGTCATTTAAGCCGATCGAACT +398
F F Q S K R Y I A Q N L E S F K P I E L

+399 AGGGTTTTTCGTCAAAGAATCTCAATTCTTCGAAACTTTACAAATGGAACACTCATTACA +458
G F F V K E S Q F F E T L Q M E H S L Q

+459 GGTTTTTTTCAACAACAACTGTAAGGCTTTGATTCCTACTAACGTGAGGAAGATTGTAGG +518
V F F N N N C K A L I P T N V R K I V G

+519 CTCACTAGTTAGTTTGTGTGTTATTACGGGGGAATATCCGATTGTTAGATATTCTGTATC +578
S L V S L C V I T G E Y P I V R Y S V S

+579 TAATCCTGTGGAAGAGGAAGATGCCCGCAATGGAAACGCCGTAGTCAACGCAAATTCTTT +638
N P V E E E D A R N G N A V V N A N S L

+639 AACAAGGTCTATTGCAAACGCTTTCCAAATAGCGATAGATACATACGCTCGCAATAATCC +698
T R S I A N A F Q I A I D T Y A R N N P

+699 AGATTTCCCTCCTCAAAACACTGAAAGACCGCGTTCCATTCTAATAATCACCGATAGAAC +758
D F P P Q N T E R P R S I L I I T D R T

+759 ACTGGAACCTTTTGCGCCAATATTACATGATTTTAGCTATCAAGCAATGGCTTATGACCT +818
L E P F A P I L H D F S Y Q A M A Y D L

+819 GGTAGCTAATGTGGACACACAAAAAGATATATATCATTATTCTGCCGAGAATGAAGCTGG +878
V A N V D T Q K D I Y H Y S A E N E A G

```
+879  TGAGCAAGAGGAAAAGGTTTCAAAATTGGTAGATTTGTATGATCCTAGCTGGATTGATTT  +938
        E  Q  E  E  K  V  S  K  L  V  D  L  Y  D  P  S  W  I  D  L

+939  GAAACATCAGCATATTATGGATGCCAACGAATATATTCAAGGAAGAATCAAGGAACTGAT  +998
        K  H  Q  H  I  M  D  A  N  E  Y  I  Q  G  R  I  K  E  L  I

+999  TGCTAAAAACCCTCTGCTGGTTGATAGATCGAACGTAAAGAATACTACCGATCTGCTGAG  +1058
        A  K  N  P  L  L  V  D  R  S  N  V  K  N  T  T  D  L  L  S

+1059 TGTCGTAGCGCACCTGAAAGATTTTGATGAAGAAAGAAGAAGGCTGATTTTGCATAAGAC  +1118
        V  V  A  H  L  K  D  F  D  E  E  R  R  R  L  I  L  H  K  T

+1119 ACTGGTAGATGAATGCCTAGGAGAGAACGCGGAAAGAAAATTAGCGGATATTTCTGCTAT  +1178
        L  V  D  E  C  L  G  E  N  A  E  R  K  L  A  D  I  S  A  I

+1179 TGAACAAAACCTATCCGGATTTGGAATGGATTTTAGTGGTGAGAAGATAAAGCATATTAT  +1238
        E  Q  N  L  S  G  F  G  M  D  F  S  G  E  K  I  K  H  I  I

+1239 CGATGATCTCTTACCAGCGTTAGCAATGAAGGAACCGACAATCTTAGATAAATTGCGTTA  +1298
        D  D  L  L  P  A  L  A  M  K  E  P  T  I  L  D  K  L  R  Y

+1299 CATTATTGCGTATGCTCTTTTCAGAGGTGGAATTATCGAGTTAGACTTCATTAAATTATT  +1358
        I  I  A  Y  A  L  F  R  G  G  I  I  E  L  D  F  I  K  L  L

+1359 GAACTTTATAGGAGTTACTCATGAACATGAAAATTTCCAGCAATATTTAAAAATATTCAG  +1418
        N  F  I  G  V  T  H  E  H  E  N  F  Q  Q  Y  L  K  I  F  R

+1419 AAATTACGATTTAATTGATTTCAAATTGATCAAAGACAAACCGAAGGATAAACCATTTCA  +1478
        N  Y  D  L  I  D  F  K  L  I  K  D  K  P  K  D  K  P  F  Q

+1479 AAAGGAATGGTTTCATGATACTTTAGTGAATGATCCAAATATCTATCACACTTCAAGGTT  +1538
        K  E  W  F  H  D  T  L  V  N  D  P  N  I  Y  H  T  S  R  F

+1539 CGTTCCCGCTGTAGGGAATATTCTTTCAAAGGTTATAGCGAATCCATTGTTATTGAGCGA  +1598
        V  P  A  V  G  N  I  L  S  K  V  I  A  N  P  L  L  L  S  E

+1599 ACAATACTTCCCATATTTAAAGGACAAGCCAATTGAGTTATTGAATGAAGAAGAATTCCA  +1658
        Q  Y  F  P  Y  L  K  D  K  P  I  E  L  L  N  E  E  E  F  Q

+1659 AGCAGGCTTAGCGAATACCTCTGCTAACTCCTCCTCATCCTTAAGGAATCCCCGTCACAA  +1718
        A  G  L  A  N  T  S  A  N  S  S  S  S  L  R  N  P  R  H  K

+1719 GGCAGCTTGGACCACGAAAAGCTCTAATATAAAAAAAAACATACCTAGACAAAGATTCTT  +1778
        A  A  W  T  T  K  S  S  N  I  K  K  N  I  P  R  Q  R  F  F

+1779 CTACTATGTTATTGGTGGTATATCAATTCCCGAAATTAAAGCTGCTTATGATCAATCAAA  +1838
        Y  Y  V  I  G  G  I  S  I  P  E  I  K  A  A  Y  D  Q  S  N

+1839 CTTGAAGAACAGAGATATCTTATTGGCAGCGACGAGATATTAACACCAACAAAATCTAGA  +1898
        L  K  N  R  D  I  L  L  A  A  T  R  Y  *

+1999 TGAATGGGAACGTTTAACAAATCCTAGAGAATTTTTCAAATTCAAGGAAGATCAGCGCCA  +1958
+2059 GCAAGTAAACCCGCCTGATTTCCTTCTAAGGAAATGAAACCGTGGCACAACCAGTCTCTC  +2018
+2119 ATGTTCATTTAAAAAGCCAAGACAATAGCCTAAACGTCTGGTACTTCTAGTCCCAAAGCA  +2078
+2179 GCAGGTTCTCTCAAGTCAGAACCACCCGAAAAAGAGAAGAAACGTAGCAAATTCTCGAG  +2137
                                                          XhoI
```

Figure 6.6 **Northern hybridisation to test for splicing of the *SEC1* transcript.**

5µg of mRNA prepared from a wild type strain (FL100) at 26°C and 37°C, and from an *rna2* mutant strain (RY26) at 26°C and 37°C, was separated by gel electrophoresis and then blotted onto nylon membrane. This filter was then probed with pMEΔO that had been linearised with *Hind*III.

Lane1 FL100, 26°C.

Lane2 FL100 , 37°C.

Lane3 RY26, 26°C.

Lane4 RY26, 37°C.

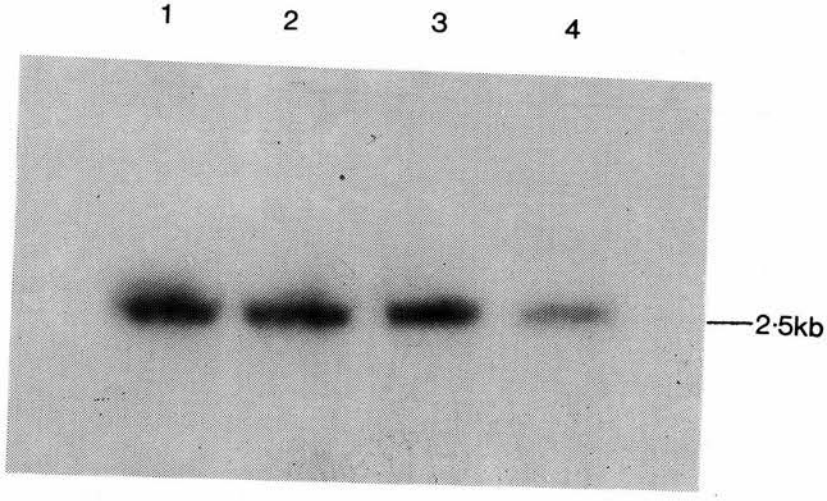

1
2
3
4
2·5kb

Figure 6.7 Comparison of Sec1p and Nim1p.

This figure was composed using the BESTFIT program of the UWGCG molecular biology software package. The alignment between residues 309 and 313 of Sec1p was done manually to give maximum similarity; (|) denotes identity and (:) a conservative change. In the region between the asparagine at position 239 of Sec1p and the valine at position 375 the two proteins share 25% identity. Those residues underlined in the Nim1p sequences are highly conserved in some protein kinases (Hunter and Cooper 1985); very few of these are present in Sec1p suggesting that Sec1p is not a protein kinase.

```
Sec1p   236 PPQNTERPRSILIITDRTLEPFAPILHDFSYQAMAYDLVANVDTQKDIYH 285
Nim1p     4 RHKNTIGVWRL....GKTLGTGSTSCVRLAKHAKTGDL.AAIKIIPIRYA 48

          286 YSAENEAGEQEEKVSKLVDLYDPSWIDLKHQHIMDANEYI.QGRIKELIA 334
           49 SIGMEILMMRLLRHPNILRLYDV.WTD..HQHMYLALEYVPDGELFHYIR 95

          335 KNPLLVDRSNVKNTTDLLSVVAHLKDFDEERRRLILHKTLVDECLGENAE 384
           96 KHGPLSEREAAHYLSQILDAVAHCHRFRFRHRDLKLENILIK...VNEQQ 142

          385 RKLADISAIEQNLSGFGMDFSGEKIKHIIDDLLPALAMKEPTILDKLRYI 434
          143 IKIADFGMATVEPNDSCLENYCGSLHYLAPEIVSHKPYRGAPADVWSCGV 192
```

Chapter Seven.

Fusion of *SEC1* coding sequences to the 3' terminus of the *lacZ* gene: purification of a ß-galactosidase-Sec1p fusion protein and its use as an immunogen.

7.1 Introduction.

The nucleotide sequence of the *SEC1* gene predicts Sec1p to have an molecular weight of approximately 73K, and to be a resident of the cytoplasm (see chapter 6). In order to confirm these characteristics and further investigate the function of Sec1p a biochemical investigation of the protein itself is required. For example, immunoprecipitation of Sec1p from wild type yeast cells will confirm the molecular weight of the protein, whereas immunofluorescence or immunoprecipitation in conjunction with subcellular fractionation will reveal the intracellular distribution of Sec1p; these techniques however, require an antibody that will specifically recognise Sec1p in a crude mixture of yeast proteins.

The first step in raising an antibody is to purify the target protein so that it can be used as an immunogen, but since no characteristic of Sec1p, other than its predicted molecular weight, are known it is not feasible to purify the protein by classic biochemical techniques. Recombinant DNA technology provides an excellent alternative: foreign genes can be fused in-frame to the 3' terminus of a gene from *E. coli* (e.g *lacZ*) which can be induced to high levels of expression, and the resultant fusion protein purified from bacterial cells and used as an immunogen (for review see Schoner *et al.*, 1985). This strategy has several advantages: the fusion protein can be made in large amounts - usually between 25-40% total cellular protein; fusion proteins are usually insoluble and invariably aggregate together to form inclusion bodies, which are easy to purify and also offer some protection against proteolysis.

In this chapter I will describe the construction and purification of a ß-galactosidase-Sec1p fusion protein. Stanley and Luzio (1984) have designed vectors specificaly for this purpose: the pEX vectors (see fig. 7.1) harbour a gene fusion of the *cro* gene of bacteriophage lambda and the *lacZ* gene of *E. coli* (see fig. 7.1), that directs the production of a cro-ß-galactosidase hybrid protein of

molecular weight 117K. The three alternative plasmids (pEX1, pEX2, or PEX3) each contain an MCS that has been placed at the 3' end of the *lacZ* gene, in different reading frames, effectively allowing any open reading frame to be fused in frame to the 3' terminus of *lacZ*. Transcription of the gene fusion is under control of the P_R promoter from bacteriophage lambda, regulated expression is therefore achieved by introducing the plasmid into a host that harbours a lysogenised phage lambda carrying the thermosensitive cI_{857} allele. At 30°C cI_{857} repressor is functional and prevents expression of fusion protein, but a shift to 42°C inactivates the repressor, allowing fusion protein to be produced up to 30-40% total cellular protein (Stanley and Luzio 1984).

7.2 Fusion of the *SEC1* gene to the 3' terminus of the *lacZ* gene.

The *Hinc*II restriction site within the coding sequence of the *SEC1* gene is unique and is compatible with the restriction sites in the MCS of the pEX vectors. The 1.5kb *Hinc*II-*Xho*I fragment encodes the carboxy-terminal 418 amino acids of Sec1p (approximate molecular weight 58K), in addition to the 3' flanking sequences; this fragment was cloned into the *Sma*I and *Sal*I restrictiion sites of pEX3, resulting in an in-frame fusion between the *SEC1* coding sequence and the 3' terminus of *lacZ*. This plasmid has been called pEXS1. A 10ml culture of *E. coli* NF1 (a phage lambda lysogen), transformed to Ap^R with pEXS1, was grown at 30°C under selection to early exponential phase; the culture was then divided into two 5ml aliquots which were incubated for a further 90min at either 42°C (to induce expression of the fusion protein) or 30°C (control). Examination of the cultures by light microcopy revealed the presence of numerous inclusion bodies inside induced cells (usually 2-3 per cell), which were completely absent from cells grown at 30°C (data not shown).

Bacterial cells were harvested by centrifugation and resuspended in 200µl water. An equal volume of sample buffer (2x) was added along with solid urea to 8M, and the cells lysed by boiling for 5min. Aliquots (40µl) from these samples were then

analysed by SDS-PAGE using a 10% (w/v) acrylamide gel (see fig. 7.2). A protein of approximate molecular weight 175K is clearly present in the induced samples, that is completely absent from the extracts of cells grown at 30°C. The size of this protein is entirely consistent with the predicted size of the cro-ß-galactosidase-Sec1p fusion protein. The amount of fusion protein observed in this experiment however, is an order of magnitude below that reported by Stanley and Luzio (1984), who observed expression upto 30% total cell protein. To increase total expression of fusion protein I extended the time of incubation at 42°C. Figure 7.3 shows the results of a time course experiment in which induction was carried out for a maximum of 6hr. The amount of fusion protein produced was seen to increase during the first four hours of induction but still not to a level equivalent to 30% of total cell protein. This may be due to the large size of the open reading frame fused to the 3' terminus of the *lacZ* gene - Stanley and Luzio (1984) observed optimal expression when DNA fragments of only 400bp were cloned into the pEX plasmids.

7.3 Large scale production and purification of fusion protein.

A 1l culture of *E. coli* NF1 (pEXS1) was grown at 30°C under selection to early exponential phase. Expression of fusion protein was induced by shifting the culture to 42°C for a further 4hr of growth. The cells were then harvested, lysed, and the inclusion bodies purified as described in chapter 2. A 1ml aliquot of purified inclusion body was applied to a 100ml G150 Sephadex column, to remove residual low molecular weight proteins and detergents prior to injection into rabbits. Fractions were collected from the column and the A_{280} determined as an estimation of protein content (fig. 7.4A). Samples were taken from fractions 12 to 17 and analysed by SDS-PAGE using a 10% acylamide gel (fig. 7.4B). The cro-ß-galactosidase-Sec1p hybrid protein is the most prominent species of protein in all four fractions; there is no evidence of contaminating proteins below molecular weight 60K, although numerous species of higher molecular weight can be seen - this is probably due to overloading of the column. Since the contaminating proteins

are of bacterial origin and are therefore unlikely to initiate production of antibodies that recognise proteins in yeast cells this purification was considered adequate.

7.4 Immunisation of rabbits with purified fusion protein.

Two female New Zealand White rabbits were each immunised with approximate 300µg of purified fusion protein. Prior to injection the immunogen was mixed with an equal volume of Freund's complete adjuvant to optimise presentation of the antigen to the rabbit immune system. The mixture was injected subcutaneously, into at least ten different positions over the back of the rabbit. Both rabbits were left untreated for a period of five weeks, after which they received further injections of material to stimulate a secondary immune response. Approximately 250µg of purified fusion protein was mixed with an equal volume of Freund's incomplete adjuvant, and injected into the rabbits using the same method described above. This treatment was repeated three times, with fourteen day intervals between each injection. Blood samples (50ml) were collected seven days after each injection and the serum tested for the presence of antibodies that recognised the fusion protein. Preliminary experiments, in which the serum obtained after the third boost has been used in a Western blot, indicate that antibodies are present that specifically recognise ß-galactosidase in a mixture of proteins (data not shown). Both rabbits have undergone another round of immunisation, the serum from this boost will shortly be tested for antibodies that recognise Sec1p in a mixture of yeast proteins.

7.5 Summary.

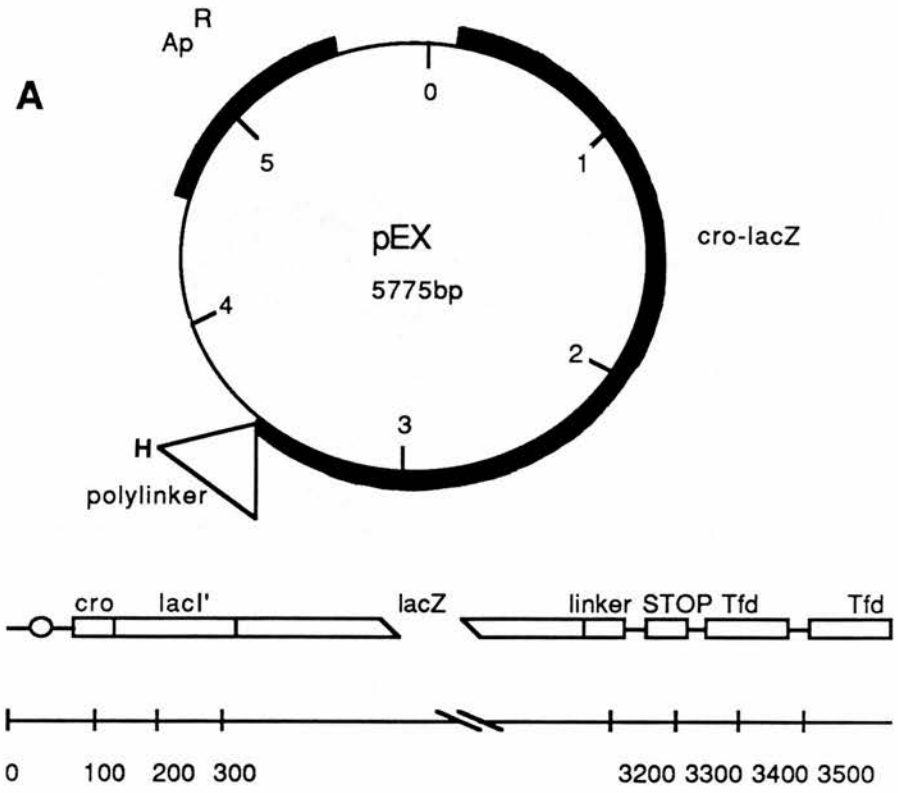
I have described the construction of a gene fusion in which a large portion of the *SEC1* gene has been placed at the 3' terminus of a *cro-lacZ* hybrid gene. In bacterial cells, this gene fusion directs the expression of a 175K cro-ß-galactosidase-Sec1p fusion protein to about 10-20% total cellular protein. Most of the fusion protein was insoluble and aggregated to form inclusion bodies. I have used differential

centrifugation and gel filtration techniques to partially purify then hybrid protein, which has been used to immunise rabbits in an attempt to raise antibodies against the *SEC1* gene product.

Figure 7.1 Map of the pEX plasmids.

Diagram A illustrates the salient features of the pEX vectors. The *cro-lacZ* gene fusion directs the expression of a 117K hybrid protein. A polylinker is located at the 3' terminus of the *lacZ* gene which can be used to insert foreign DNA fragments in frame with ß-galactosidase. Immediately 3' to the polylinker is a synthetic oligonucleotide (STOP) containing stop codons in all open reading frames, and a transcription termination fragment (Tfd) taken from phage Tfd. See Stanley and Luzio (1984) for more details.

Diagram B shows the polylinker of the three pEX vectors, with the *lacZ* reading frame and restriction sites in the polylinker marked. Plasmid pEXS1 was formed by cloning the 1.5kb *Hinc*II-*Xho*I fragment, carrying *SEC1* coding sequences into the *Sma*I and *Sal*I sites of pEXS3.



B

pEX1

BamHI PstI
SmaI SalI HindIII

GCC CGG GGA TCC GTC GAC CTG CAG CCA AGC TTG CTG ATT GAT

A R G S V D L Q P S L L I D

pEX2

EcoRI BamHI PstI
SmaI SalI HindIII

GAA TTC CCG GGG ATC CGT CGA CCT GCA GCC AAG CTT GCT GAT

Q F P G I R R P A A K L A D

pEX3

BamHI PstI
SmaI SalI HindIII

GAA TTA ATT CCC GGG GAT CCG TCG ACC TGC AGC CAA GCT TGC

Q L I P G D P S T C S Q A C

Figure 7.2 **Expression of the cro-ß-galactosidase-Sec1p fusion protein in *E. coli*.**

E. coli strain NF1 was transformed to Ap^R with pEXS1 and grown at 30°C to early exponential phase (A_{600}=0.3). To induce expression of fusion protein, cultures were shifted to 42°C and incubated for a further 40min; cells were harvested, lysed and prepared for SDS-PAGE analysis as described elsewhere. Proteins were visualised by staining with Coomassie blue.

Lane 1 Standard proteins

Lane 2 Extract from *E. coli* NF1 grown at 42°C.

Lane 3 Extract from *E. coli* NF1 transformed to Ap^R with pEXS1

Standard proteins: Myosin (200K), ß-galactosidase (116K), Phosphorylase b (92.5K), BSA (69K), Ovalbumin (46K), Carbonic anhydrase (30K), Lysozyme (14.3K).

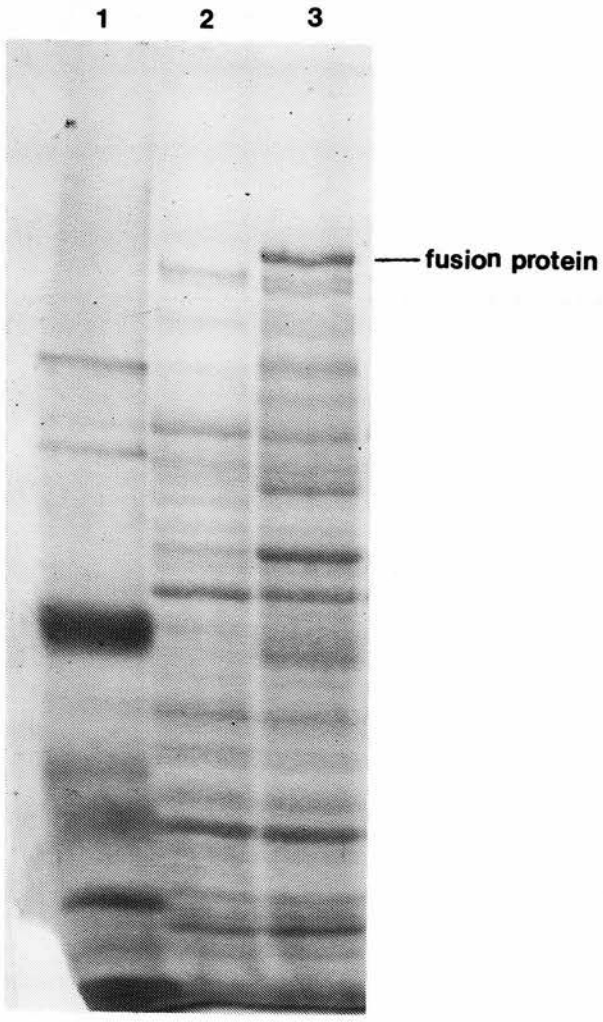

1
2
3
fusion protein

Figure 7.3 **Improved expression of cro-ß-galactosidase-Sec1p fusion protein in *E. coli*.**

A 50ml culture of *E. coli* NF1 transformed to Ap^R with pEXS1 was grown at 30°C to early exponential phase (A_{600}=0.3), at which point the culture was shifted to 42°C. Aliquots of 5ml were removed at T= 0,1,2,3,4,5, and 6 hours and processed for SDS-PAGE as described elsewhere. Proteins were visualised by staining with Coomassie blue.

Lane 1 Standard proteins (as in fig. 7.4)

Lane 2 6 hr induction at 42°C

Lane 3 5 hr induction at 42°C

Lane 4 4 hr induction at 42°C

Lane 5 3 hr induction at 42°C

Lane 6 2 hr induction at 42°C

Lane 7 1 hr induction at 42°C

Lane 8 No induction.

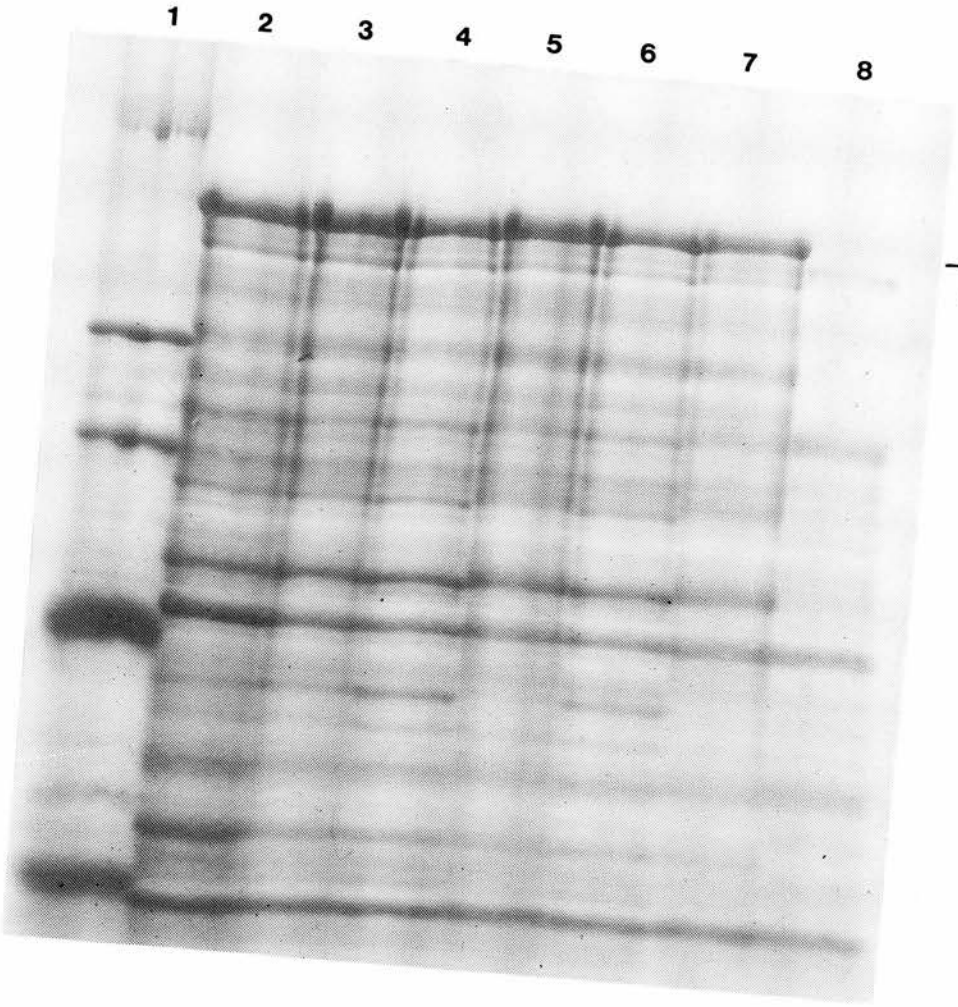

1
2
3
4
5
6
7
8
—fusion protein

Figure 7.4 **Purification of cro-ß-galactosidase-Sec1p fusion protein.**

A. Inclusion bodies containing the fusion protein were first partially purified from bacterial cells by differential centrifugation. Approximately one third of this material was applied to a G150 Sephadex gel filtration chromatography column. Fractions of 0.7ml were collected every two minutes and the A_{280} determined as an estimation of the protein concentration. In total, thirty seven fractions were collected.

B. Aliquots (40µl) from column fractions 12 to 17 were analysed by SDS-PAGE using a 10% acrylamide gel. Proteins were visualised by staing with coomassie blue.

Lane 1 Standard proteins (as in fig. 7.4)

Lane 2 Extract from *E. coli* NF1 grown at 42°C.

Lane 3 Extract from *E. coli* NF1 transformed with pEXS1 grown at 42°C.

Lane 4 cro-ß-galactosidase fusion protein purified by differential centrifugation.

Lane 5 Column fraction 12 (40µl)

Lane 6 Column fraction 13 (40µl)

Lane 7 Column fraction 14 (40µl)

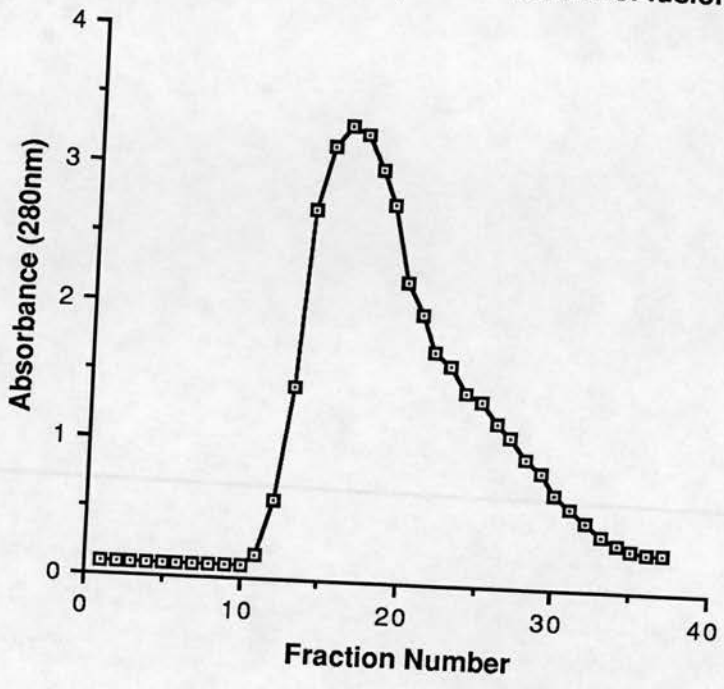
Lane 8 Column fraction 15 (40µl)

Lane 9 Column fraction 16 (40µl)

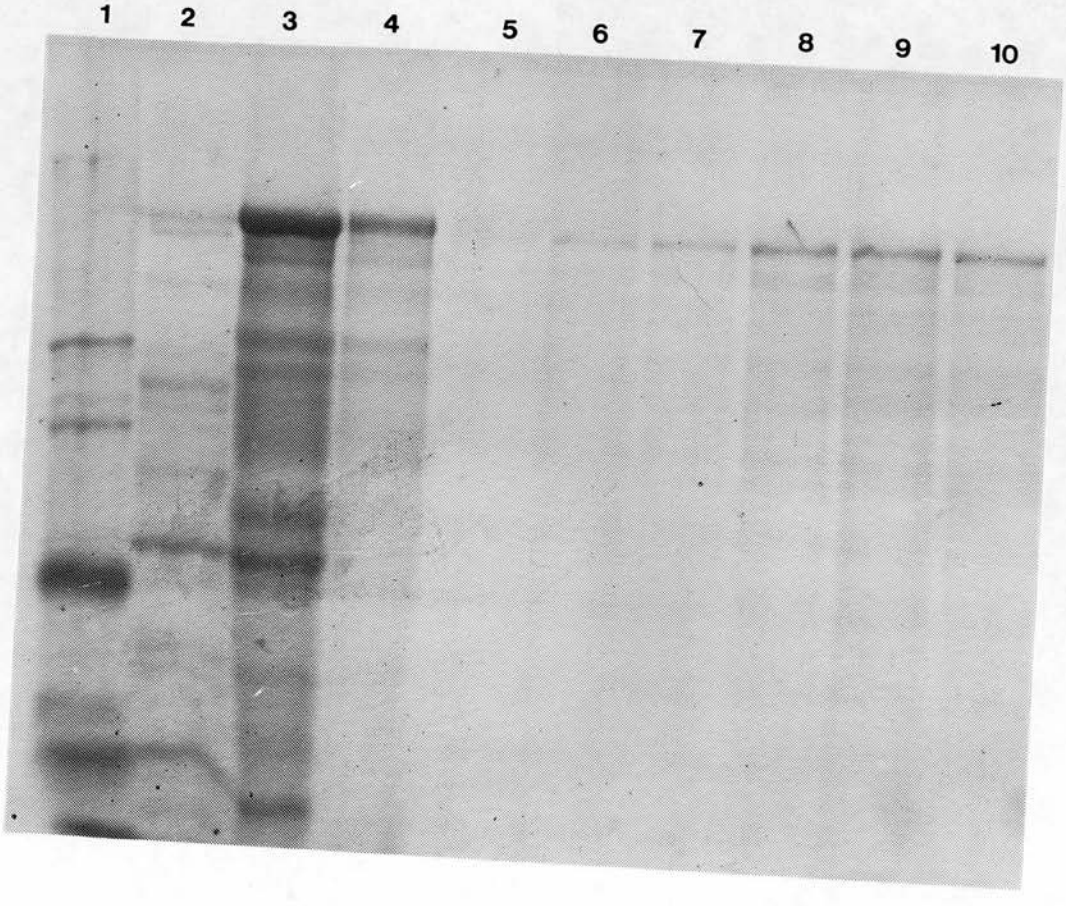
Lane 10 Column fraction 17 (40µl)

A

Gel filtration on G150 Sephadex column of fusion protein.



B



Chapter Eight.

The cloned fragment of genomic DNA also carries the *SEC5* gene: mapping of the *SEC5* gene and investigation of its relationship with the *SEC1* gene.

8.1 Introduction.

The *SEC1* gene is located on the long arm of chromosome IV, in a tightly linked cluster of genes including *CDC37*, *SEC5*, and *SEC7*. Previous genetic analysis has determined the arrangement of these genes within the cluster (see Mortimer and Schild 1985); the *SEC5* gene has been found to lie adjacent to the *SEC1* gene. In this chapter I will demonstrate that the genomic fragment of pEDB1 also carries a second gene, independent of the *SEC1* gene, that can complement the *sec5-24* mutation.

Mutations in the *SEC5* gene result in a phenotype similiar to that displayed by a *sec1-1* mutant, ie: a late block of the secretion pathway, characterised by an intracellular accumulation of secretory vesicles, and a cessation of endocytosis at 37°C. It is possible that this is a reflection of some overlap of function between the *SEC1* and *SEC5* gene products, perhaps because the two genes have evolved from a common ancestral sequence. I will describe experiments to demonstrate genetic interaction between the *SEC1* and *SEC5* genes, suggesting functional overlap between Sec1p and Sec5p, but I also show that this relationship is not due to similarities in the sequence (and therefore the products) of the *SEC1* and *SEC5* genes.

8.2 The 10.6kb genomic fragment of pEDB1 carries a gene that can complement the *sec5-24* mutation.

MEY511 (*sec5-24, leu2-3,112*) was transformed to leucine prototrophy with pEDB1, and tested for the ability to grow at 37°C. All transformants tested grew at 37°C, indicating that pEDB1 carries a gene that can complement the *sec5-24* mutation. Plasmid pEDB16, which carries the *SEC1* gene could not support growth of MEY511 at 37°C. This clearly demonstrates the presence of a second gene, distinguishable from the *SEC1* gene, in the genomic fragment of pEDB1 that can complement the *sec5-24* mutation. Since the *SEC1* and *SEC5* genes have been shown to be tightly linked in this fashion this gene is almost certainly the authentic *SEC5*

gene, although formal genetic proof of this is yet to be obtained. Further complementation experiments revealed that none of the pEDB1 derivatives described in chapter 3, namely: pEDB14, pEDB15, and pEDB18, were capable of supporting growth of MEY511 at 37°C, thereby suggesting that the *SEC5* gene could not be subcloned on a single *Hind*III fragment.

8.3 Mapping of the *SEC5* gene by Tn5 mutagenesis; and subcloning of the gene.

In order to locate the *SEC5* gene within the 10.6kb genomic fragment, pEDB1 was analysed by Tn5 mutagenesis in exactly the same way as described in chapter 2. This project was carried out in collaboration with Dr Alan Boyd, but for completeness I have presented these results in fig 8.1. Of the 8 insertions analysed one, p3-11, was observed to completely abolish the ability of pEDB1 to complement the *sec5-24* mutation, and is therefore presumably within the *SEC5* gene. This insertion is at least 3.5kb away from the *SEC1* gene, and thus confirms the existence of two independent genes.

I have used one of the Tn5 derivatives described above to subclone the *SEC5* gene. As described in chapter 2 the insertion of the Tn5 molecule into pEDB1 results in the introduction of new restriction sites. Plasmid p3-2 contains a transposon insertion to the right of the *SEC5* gene (in between *SEC5* and *SEC1*); using the *Bam*HI site of the Tn5 molecule and the *Bam*HI site at the junction of the genomic DNA and vector DNA sequences, all the yeast genomic DNA to the right of the transposon was deleted (see fig 8.1). The resulting plasmid has been termed pEDB32, and when introduced into MEY511 can fully support its growth at 37°C.

8.4 Genetic interaction between the *SEC1* and *SEC5* genes.

The suggestion that the *SEC1* and *SEC5* genes might have arisen from a common ancestral sequence also implies that the respective polypeptides will be similiar in sequence and perhaps function. The experiment described in section 8.2 however, demonstrates that the *SEC1* gene cannot support the growth of a *sec5-24* mutant at 37°C, even when present in multicopy. The same question was asked of the *SEC5* gene, ie. could it suppress the *sec1-1* mutation at 37°C. In summary, ABY12 cells transformed to leucine prototrophy by pEDB32 were found to be incapable of growth at 37°C, indicating that the *SEC5* gene cannot suppress mutations in the *SEC1* gene.

As described in the Introduction, Salminen and Novick (1987) observed that duplication of the *SEC4* gene suppressed numerous late acting *sec* mutations at 33.5°C (an intermediate temperature that is lethal for the parental strains) but not at 37°C. I therefore proceeded to test whether at 33.5°C, the *sec1-1* mutation could be suppressed by multiple copies of the *SEC5* gene and similarly, if the *sec5-24* mutation could be suppressed by multiple copies of the *SEC1* gene. The results are summarised in table 8.1. Considering complementation of *sec5-24* by *SEC1* first: the recipient strain (MEY511) is incapable of growth at 33.5°C; growth is completely restored when MEY511 is transformed with pEDB1 or pEDB32 (positive controls) but not with YEp13 (negative control). However, MEY511 cells transformed with pEDB16 (the *SEC1* gene) also grew at 33.5°C, and moreover, did not appear significantly different from MEY511 cells transformed with pEDB32, as judged by colony size. To confirm that this effect was due to the *SEC1* gene, the Tn5 mutagenised derivatives of pEDB16 (pTN, see chapter 4) were tested for the ability to complement the *sec5-24* mutation at 33.5°C: the four plasmids which contain Tn5 insertions within the *SEC1* gene (pTN18, pTN26, pTN57, and pTN64) did not allow growth of MEY511 at 33.5°C (data not shown). Multiple copies of the *SEC1* gene therefore cannot suppress mutations in the *SEC5* gene at 37°C but can do so at 33.5°C. In direct contrast, it was found that increasing the number of copies of the

SEC5 gene had no effect upon the growth of a *sec1-1* mutant at either 37°C or 33.5°C. The functional relationship that exists between *SEC1* and *SEC5* is therefore unidirectional.

8.5 Southern hybridisation experiments to further examine the relationship between *SEC1* and *SEC5*.

One possible explanation for the genetic interaction between the *SEC1* and *SEC5* genes is a similarity in the sequences of the two genes. I have used Southern hybridisation experiments to test this hypothesis; if *SEC1* and *SEC5* do have related sequences then a DNA fragment carrying the *SEC1* gene will hybridise to both *SEC1* and *SEC5* sequences in pEDB1.

Plasmid pEDB1 was cleaved with *Eco*RI, the fragments separated by gel electrophoresis, transferred to nitrocellulose, and then probed with pHX18. Two *Eco*RI fragments of 3.2kb and 1.2kb should hybridise to this probe since they have identical sequences. If the *SEC5* gene has any sequence similarity to the *SEC1* gene, additional fragments would also be detected. The hybridisation reaction was carried out under conditions of reduced stringency (6xSSC, 50°C) thereby allowing approximately 25% mismatch between sequences. The results are presented in fig 8.2: the fragments of 3.2kb and 1.2kb are clearly visible but there is no evidence for any additional fragments hybridising to the probe, even following prolonged exposure (data not shown). If the two genes do share related sequences therefore, they are not detectable by this method. I have almost completed sequencing the *SEC5* gene: to date an incomplete open reading frame of 1560bp, sufficient to encode a polypeptide of 520 residues has been identified; no similarities between this sequence and the *SEC1* sequence have been found (data not shown).

8.6 Genetic interactions between the *SEC1* and /or the *SEC5* genes and other late-acting *SEC* genes.

I have extended the genetic analysis described in section 8.4 to include many of the other late acting *sec* mutations. Plasmids pEDB1, pEDB16, and pEDB32 were tested for the ability to complement the *sec2-56, sec3-2, sec6-4, sec8-6, sec9-4, sec10-2, and sec15-1* mutations at either 33.5°C or 37°C; complementation was scored by the ability of transformed strains to form single colonies under restrictive conditions (see table 8.2). Increasing the number of copies of the *SEC1* gene was found to suppress the *sec3-2, sec9-4,* and *sec15-1* mutations at 33.5°C, whereas multiple copies of the *SEC5* gene suppressed the *sec3-2, sec8-4*, and *sec10-2* mutations at 33.5°C. Complementation of the *sec10-2* mutant is of particular interest: at 33.5°C, increased copies of the *SEC1* gene alone (pEDB16) cannot suppress the *sec10-2* mutation, but increase copies of the *SEC5* gene alone (pEDB32) can; however, when the copy number of both the *SEC5* and *SEC1* genes is increased (pEDB1) the *sec10-2* mutant cannot grow at 33.5°C. In this experiment therefore, Sec1p must somehow modulate the activity of Sec5p.

One mutation, *sec3-2*, can also be complemented at 37°C when pEDB1 is introduced into the mutant strain but not when pEDB16 or pEDB32 are tested, suggesting that under full restrictive conditions multiple copies of both the *SEC1* and *SEC5* genes are required to support growth of a *sec3-2* mutant. To confirm that this effect was indeed due to the *SEC1* and *SEC5* genes, and not some other unidentified gene present on the genomic fragment of pEDB1, four of the Tn5 derivatives described in section 8.2 were tested for the ability to complement the *sec3-2* mutation at 37°C. The four derivatives chosen (see fig 8.1) contained a Tn5 molecule inserted into the *SEC1* gene (p2-6), or the *SEC5* gene (p3-11), or the region lying between the *SEC1* and *SEC5* genes (p1-6, p3-11). As predicted the latter two derivatives still retained the ability to complement the *sec3-2* mutation at 37°C and the Tn5 insertion into the *SEC5* gene abolished the complementing activity of pEDB1. Surprisingly, the Tn5

insertion into the *SEC1* gene, that abolishes the ability of pEDB1 to complement the *sec1-1* mutation at 37°C, had no effect on complementation of the *sec3-2* mutation. However, this Tn5 insertion is positioned towards the 3' terminus of the *SEC1* gene, and thus a large truncated *SEC1* polypeptide could be produced; but since this has lost its biological activity (as defined by complementation of the *sec1-1* mutation at 37°C) the most probable way in which it could exert this effect on complementation of the *sec3-2* mutation is by interaction with a second protein, possibly Sec5p. Further analysis of Tn5 insertions into 5' regions of the *SEC1* gene are required to confirm this hypothesis, and rule out the formal possibility that there is a further gene, carried on the genomic fragment of pEDB1, that is in part responsible for complementation of the *sec3-2* mutation.

8.8 Summary.

Previous genetic analysis has mapped the *SEC1* gene adjacent to the *SEC5* gene on the long arm of chromosome IV. I have demonstrated that pEDB1, isolated from the Nasmyth library by its ability to complement the *sec1-1* mutation at 37°C, is also capable of complementing the *sec5-24* mutation at 37°C. The region of DNA that confers this ability has been located by Tn5 mutagenesis and has been found to lie approximately 3.5kb away from the *SEC1* gene. Formal genetic proof that this gene is the authentic *SEC5* gene is currently being obtained.

Complementation analysis has revealed a genetic relationship between the *SEC1* and the *SEC5* genes: at a restrictive temperature of 33.5°C multiple copies of the *SEC1* gene can suppress the *sec5-24* mutation, but this relationship is unidirectional since increasing the number of *SEC5* genes does not suppress the *sec1-1* mutation. The biochemical basis for this interaction is not known, although Southern hybridisation experiments and partial sequence analysis make it very unlikely that it reflects similarity in the sequences of the *SEC1* and *SEC5* genes.

I have extended the genetic interaction described above to include other late-acting *sec* mutations, and have demonstrated that increasing the number of the *SEC1* and/or the *SEC5* genes can suppress the *sec3-2, sec8-6, sec9-4, sec10-2, and sec15-1* mutations at 33.5°C and also the *sec3-2* mutation at 37°C. Circumstantial evidence has also been obtained which suggests that Sec1p may interact directly with Sec5p. This confirms and extends the observations of Salminen and Novick (1987), who described similiar genetic interaction between the *SEC4* gene and other late acting *sec* mutations. The possible implications of this type of interaction are discussed in chapter 9.

Figure 8.1 **Localisation of the *SEC5* gene.**

Plasmid pEDB1 was mutagenised with the transposon Tn5 as described in chapter 2; the position of Tn5 insertion was determined by restriction mapping and then several derivatives were tested for the ability to complement the *sec5-24* mutation at 37°C. MEY511 was transformed to leucine prototrophy with the mutagenised plasmids, and then tested for the ability to form single colonies at 37°C. One insertion was identified (▼) that completely abolished the complementing activity of pEDB1; this was flanked by numerous insertions that had no effect (▽).

Insertion of a Tn5 molecule introduces new restriction sites, the *Bam*HI site of one Tn5 derivative (p3-2) was used in conjunction with the *Bam*HI site at the righthand boundary of the insert and vector sequences, to delete all the DNA to the right of the *SEC5* gene. The resulting plasmid, pEDB32, still retains the ability to complement the *sec5-24* mutation at 37°C.

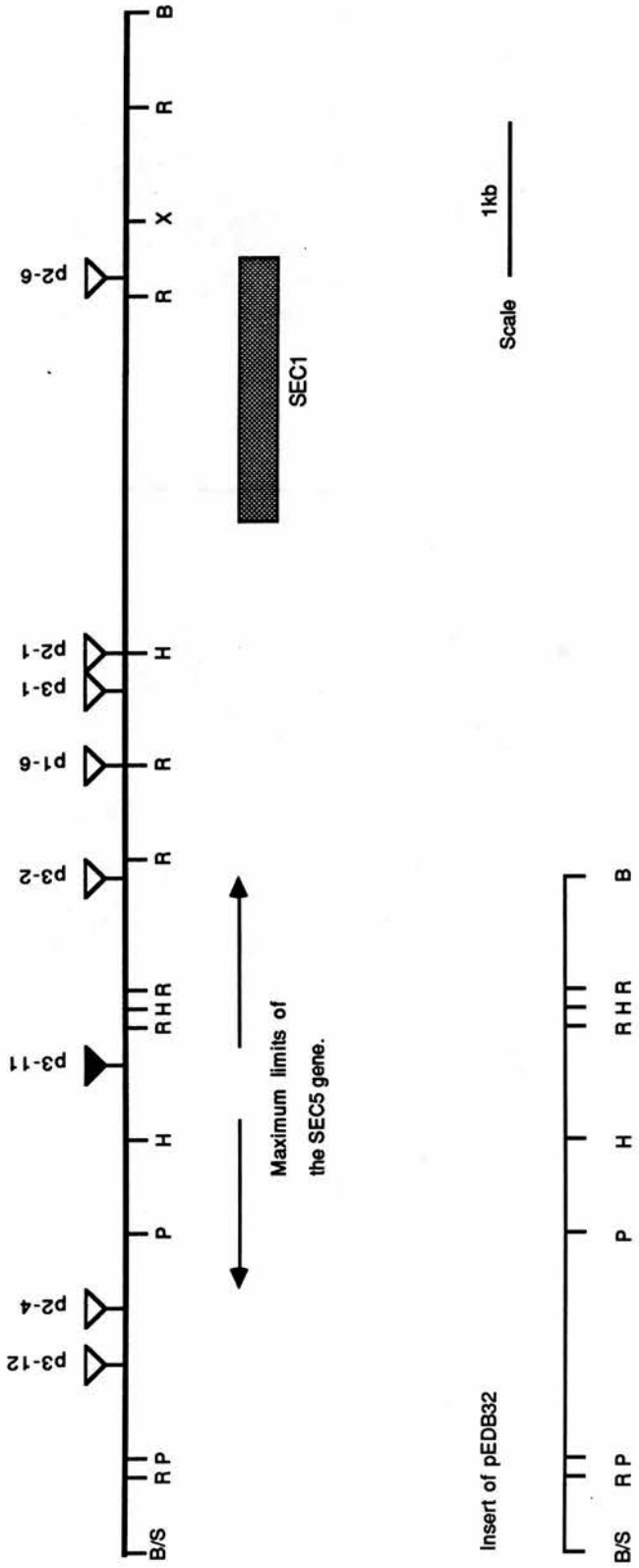

B/S
R P
p3-12
p2-4
P
H
R H R
p3-11
p3-2
R
p1-6
R
p3-1
p2-1
H
R
X
R
p2-6
B
SEC1
Maximum limits of
the SEC5 gene.
Scale
1kb
Insert of pEDB32
B/S
R P
P
H
R H R
B

Table 8.1 Genetic interaction between the *SEC1* and *SEC5* genes.

Plasmids pEDB1, pEDB16, pEDB32, and YEp13 were used to transform ABY12 and MEY511 to leucine prototrophy. Transformants were selected at 26°C by growth on minimal medium in the absence of leucine. Four transformants were then streaked onto fresh minimal medium and incubated at 26°C, 33.5°C, and 37°C for 48hrs -72hrs. Complementation was scored by the ability to form single colonies; (+) represents complementation and, (-) no complementation.

Complementation of sec5-24 by the SEC1 gene.

	Temperature at which transformants were tested for growth.		
	26°C	33.5°C	37°C
YEp13	+	-	-
pEDB1	+	+	+
pEDB16	+	+	-
pEDB32	+	+	+

Complementation of sec1-1 by the SEC5 gene

	Temperature at which transformants were tested for growth.		
	26°C	33.5°C	37°C
YEp13	+	-	-
pEDB1	+	+	+
pEDB16	+	+	+
pEDB32	+	-	-

Figure 8.2 **Southern hybridisation experiment to test for sequence similiarities between the *SEC1* and *SEC5* genes.**

Plasmid pEDB1 was cleaved with *Eco*RI, to produce 8 fragments of 0.65, 1.2, 1.3, 2.4, 3.2, 3.25, 4.2, and 4.3kb (data not shown). The fragments were separated by gel electrophoresis and transferred to a nitrocellulose filter. This was probed, under conditions of low stringency (6xSSC, 50°C) with pHX18 that had been radiolabelled with [α^{32}]P-dATP. Under these conditions only two fragments were seen to hybridise to the probe, and both correspond to *Eco*RI fragments which carry *SEC1* sequences. No additional fragments were seen to hybridise to the probe, even after prolonged exposure.

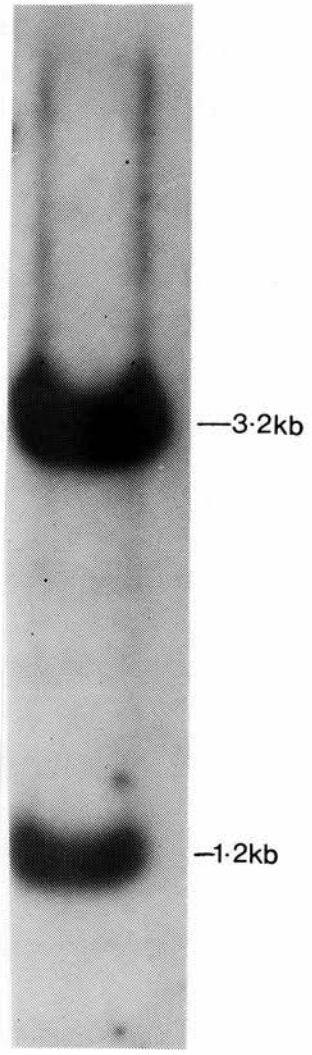

—3·2kb
—1·2kb

Table 8.2 **Genetic interactions between the *SEC1* and/or *SEC5* genes and other late-acting *SEC* genes.**

Plasmids pEDB1, pEDB16, pEDB32, and YEp13 were introduced into numerous strains. Transformants were selected by growth on minimal medium, in the absence of leucine, at 26°C. Four transformants were then streaked onto fresh selective medium and tested for the ability to form single colonies at 26°C, 33.5°C, and 37°C. Complementation was scored after 48hrs incubation.

Plasmids introduced into strains and temperatures at which growth was tested

STRAIN	YEp13			pEDB16			pEDB32			pEDB1		
	26°C	33.5°C	37°C	26°C	33.5°C	37°C	26°C	33.5°C	37°C	26°C	33.5°C	37°C
MEY230 (sec2-56)	+	+	-	+	+	-	+	+	-	+	+	-
MEY301 (sec3-2)	+	-	-	+	+	-	+	+	-	+	+	+
MEY613 (sec6-4)	+	-	-	+	-	-	+	-	-	+	-	-
MEY836 (sec8-6)	+	+/-	-	+	+/-	-	+	+	-	+	+	-
MEY908 (sec9-4)	+	-	-	+	+	-	+	-	-	+	+	-
MEY1007 (sec10-2)	+	-	-	+	-	-	+	+	-	+	-	-
MEY1503 (sec15-1)	+	+/-	-	+	+	-	+	+/-	-	+	+	-

Chapter Nine.

Discussion and Conclusions.

9.1 Discussion.

Transport of proteins from the endoplasmic reticulum to the cell surface in *S. cerevisae* requires at least 23 *SEC* gene products. In this thesis I have described the isolation of a fragment of genomic DNA which carries both the *SEC1* and *SEC5* genes both of which are thought to encode proteins that function during the late stages of the secretory pathway, where secretory vesicles fuse with the plasma membrane. In a previous report, Salminen and Novick (1987) demonstrated extensive genetic interaction between those *SEC* genes whose products function in this area of the pathway: duplication of the *SEC4* gene can suppress mutations in six other late acting *SEC* genes, including the *sec1-1* and *sec5-24* mutations. In this thesis I have demonstrated further genetic interactions which extend these observations: at 33.5°C multiple copies of the *SEC1* gene can suppress the *sec3-2, sec5-24, sec9-4*, and *sec15-1* mutations, whereas increasing the number of copies of the *SEC5* gene can suppress the *sec3-2, sec8-2*, and *sec10-2* mutations; one mutation, *sec3-2* was also suppressed at 37°C when the copy number of both genes was increased. It is important to emphasise that the genetic interactions described by Salminen and Novick (1987) and in this thesis result from overexpression of *SEC* gene products. Suppression of this type can reflect a similarity in the structure and function of the gene products. Thus, *S. cerevisiae* has two genes that encode α–tubulin, *TUB1* and *TUB3*; the *TUB1* gene is essential for cell viability but can be dispensed with if the *TUB3* gene is present in multiple copies (Schatz *et al.*,1986a,b). It seems unlikely however, that so many *SEC* genes will encode proteins so closely related in structure and function. In fact the observation that the *SEC1* and *SEC4* genes are each essential in their own right suggests that they do not have overlapping functions, but moreover I have demonstrated that there is no sequence similarity between the *SEC1*, *SEC5* or *SEC4* genes, despite the observed interactions.

In order to explain the interactions that they observed, Salminen and Novick (1987) proposed that there may be multiple pathways in secretion and that Sec4p may

function on a pathway that is parallel to that one on which the interacting *SEC* gene products function. In this model overexpression of Sec4p would compensate for a partial defect on an alternative pathway, while defects on both pathways would be synergistically deleterious and therefore lethal at all temperatures. This model is not consistent with the genetic interactions described in this thesis. As an alternative hypothesis, I propose that genetic interactions of this type are observed where the activity of one *SEC* gene product is dependent upon the activity of another; in this view the late acting *SEC* gene products therefore only acquire function if they either form a complex with or are otherwise activated by another *SEC* protein(s) (see fig. 9.1 and legend for full description).

A clue to what Sec1p may be interacting with is provided by its resemblance to the *nim1*$^+$ gene product (Nim1p) of *Schizosaccharomyce pombe*. By sequence homology Nim1p is proposed to be a protein kinase. Residues 239 to 375 of Sec1p share 25% identity with residues 7 to 136 of Nim1p; this region of Nim1p also contains sequence motifs that correlate with protein kinase activity, but very few of these are present in Sec1p indicating that Sec1p is not a protein kinase. Nim1p is thought to participate in the control of cell division since the *nim1*$^+$ gene was isolated by its ability to suppress *cdc25*$^+$ mutations when present in multiple copies. Russell and Nurse (1987) postulate that Nim1p is a member of a regulatory network that controls cell division (fig. 9.2); in this model the *cdc2*$^+$ gene product (Cdc2p) is a master regulator of mitosis whose function is rate limiting. The *cdc25*$^+$ and *wee1*$^+$ gene products (Cdc25p and Wee1p respectively) exert control over the activity of Cdc2p: Cdc25p stimulates Cdc2p mitotic function and is therefore an inducer of mitosis, whereas Wee1p counteracts Cdc2p mitotic function and is thus an inhibitor of mitosis - the balance of Cdc25p and Wee1p is therefore an important control element of mitosis. Because the *nim1*$^+$ gene can compensate for mutations in the *cdc25*$^+$ gene, Nim1p is therefore thought to be an inducer of mitosis and could function as a negative regulator of Wee1p or by modification of Cdc2p to prevent it responding to Wee1p.

The similarity of the two proteins could indicate that Sec1p is able to bind a substrate protein(s) which is also recognised by a Nim1p-like protein. I have presented two hypotheses to describe such an interaction (fig. 9.3): model A suggests that Nim1p recognises a substrate and subsequently phosphorylates it, resulting in a slight conformational change of the substrate which allows recognition and binding by Sec1p; model B implies that the substrate can interact with Sec1p without prior phosphorylation, so that competition for substrate would exist between Nim1p and Sec1p. Two predictions made by both models are that *S. cerevisiae* will possess a homologue of the *S. pombe nim1*$^{+}$ gene, and that Sec1p will interact directly with other proteins. It is likely that *S. cerevisiae* will indeed have a *nim1*$^{+}$ homologue since genetic analysis has demonstrated that the *CDC28* gene of *S. cerevisiae* is homologous to the *cdc2*$^{+}$ gene of *S. pombe*, in fact the two genes can substitute for each other implying that the two yeasts have similiar cell cycle control mechanisms (Beach *et al.*, 1982). In support of the second prediction I have demonstrated extensive genetic interaction between the *SEC1* gene and other *SEC* genes, which are most readily explained by interaction of Sec1p with other *SEC* gene products.

The similarity between Sec1p and Nim1p provides a connection, albeit tenuous, between a cell cycle control circuit and secretion; there are clear indications that some such connection must exist. Cell division in *S. cerevisiae* is asymmetrical, such that newly formed daughter cells are smaller in size and take longer to divide than the parental cells they originated from (Hartwell and Unger 1977). It has been proposed that there is a critical size which must be achieved prior to initiation of a division cycle, ie. daughter cells are required to be of similar size to the parental cell before division. During this period of growth there is no bud development and new material is probably deposited over the whole of the cell surface. On reaching the critical size cells enter into a division cycle and develop a bud. The onset of budding is anticipated by the appearance of vesicles below the plasma membrane, and an array of microtubules extending from the spindle pole body on the nucleus to the presumptive site of bud emergence (for review see Wheals 1987). Throughout the

subsequent budding phase of the cell cycle all secretion and membrane expansion are restricted to the developing bud. Control systems must therefore exist which enable the cell to regulate fusion of secretory vesicles with the plasma membrane in both a spatial and temporal manner. The late acting *SEC* gene products may be elements of such regulatory mechanisms.

The biochemical mechanism that underlies the switch from de-localised to polarised membrane expansion is unknown, but ultimately it must involve an efficient cytoskeletal system for targetted transport of secretory vesicles to a specific region of the plasma membrane that is competent for membrane fusion. Evidence for a relationship between late-acting *SEC* gene products and the cytoskeleton is beginning to emerge. Thus, two *SEC* gene products have been visualised in cells by immunofluorescence techniques. The *SEC4* gene product is observed in association with both secretory vesicles and the plasma membrane (Goud *et al.*, 1988). The *SEC15* gene product displays a intracellular distribution similar to that of actin, that is, it is observed in patches at the periphery of the cell with several cables extending from these patches to the interior of the cell and through the neck of the bud (Antti Salminen, personal communication). The cytoskeleton has previously been implicated to function in the late stages of the secretion pathway from the phenotype of the *act1-1* and *act2-1* mutations (temperature sensitive actin mutations): under restrictive conditions mutant cells exhibit an intracellular accumulation of invertase and secretory vesicles in similar fashion to late-blocking *sec* mutants. It is possible that the interacting group of late-acting *SEC* genes encode proteins which are cytoskeletal elements or which promote association of secretory vesicles with the cytoskeletal network.

In relation to this, a further clue to the function of Sec1p may be provided by erythrocytes, which also have an extensive cytoskeletal network that forms a shell under the entire plasma membrane (for review see Backman 1988, fig. 9.4). Many of the interactions shown in fig. 9.4 are influenced by

phosphorylation/dephosphorylation reactions. For example, protein 4.9 has the ability to bundle actin microfilaments into filaments but this ability is lost when protein 4.9 is phosphorylated (Husain-Chisti *et al.*, 1988). Alternatively, ankyrin is proposed to anchor the cytoskeleton to the plasma membrane by interaction with band 3; phosphorylation of ankyrin reduces its affinity for band 3 (Cianci *et al.*, 1988). Thus the integrity of the cytoskeletal network breaks down when certain of its components are phosphorylated. Many of the proteins shown in fig. 9.3, including band 4.9 and ankyrin have been identified in several non-erythrocyte cells (e.g see Anderson *et al.*, 1988), and one protein, spectrin, has been identified in *S. cerevisae* (Eli Orr, personal communication). Phosphorylation of these and other proteins could therefore be a general mechanism by which bundling of actin microfilaments and their association with the plasma membrane is controlled. It is interesting to speculate that Sec1p could block phosphorylation of a cytoskeletal protein, such as a protein similar to protein 4.9, thereby maintaining the integrity and organisation of the actin cytoskeleton. Clearly, most of the interesting questions about Sec1p function remain to be answered but these speculative ideas provide a framework for the design of future experiments.

9.2 Conclusions.

The *sec* mutants have provided an invaluable tool for investigations of protein secretion from *S. cerevisae*; they have allowed the secretory pathway to be genetically dissected and biochemically characterised. The fact that only 23 complementation groups have been identified however, many of which have only one member, suggests that this is not a complete representation of the gene products required for transport of proteins from the ER to the cell surface. This is particulary true of the Golgi complex where only two genes have been identified whose products directly affect transport through the organelle, yet it is perhaps the most intricate component of the secretory pathway. Further mutants could no doubt be isolated by extending the mutant hunt of Novick and Schekman (1980), but it is

likely that many genes will remain elusive because of the characteristics of the mutant selection protocol. The proceedures employed by Novick and Schekman (1980) necessitate that mutant strains remain viable at 37°C for up to three hours; for many mutants this may not be realistic. Actin has been implicated in secretion by the fact that mutations in the actin gene lead to a phenotype similiar to that of a late *sec* mutant and one would therefore predict that the actin gene could have been isolated in the *sec* mutant hunt. However, at 37°C the two actin mutations result in an 88% loss in viability after four hours of incubation, suggesting that such mutants would not have survived the mutant selection protocol. It was anticipated that analysis of the late acting *SEC* genes and their products would provide an insight into some of the mechanisms employed during fusion of secretory vesicles with the plasma membrane. But what is beginning to emerge from preliminary investigations is that these genes encode proteins which facilitate intracellular transport of secretory vesicles. It may be necessary therefore to resort to more conventional biochemical techniques to uncover such proteins before the genetic techniques available with *S. cerevisae* realise their true potential.

Figure 9.1 **Interaction between the late-acting *SEC* gene products.**

In this model the activity of one *SEC* protein is dependent upon another *SEC* protein. Protein B is a substrate for protein A, which functions to either activate protein B to B' or associates with B to form an AB complex. Activated B' or the AB complex subsequently interacts with protein C to produce activated C' or an ABC complex. In similar fashion C' or ABC acts upon D to produce either D' or an ABCD complex.

Removal of any component in this pathway, e.g by a temperature sensitive mutation, would interrupt the chain of events and ultimately result in loss of function. Many of the genetic interactions described in this thesis and by Salminen and Novick (1987) are observed at 33.5°C. At this temperature, proteins encoded by mutant *sec* alleles may retain activity, but at reduced levels. Such deficiencies could be compensated for by overexpression of an interacting protein e.g defects in protein A could be overcome by overexpression of protein B, thereby favouring the formation of either B' or the AB complex. A similar argument can be used to explain some of the genetic interactions observed at 37°C. It is known that the *sec4-8* mutant gene encodes a temperature sensitive protein that retains activity at 37°C, since overexpression of the *sec4-8* gene product can complement the *sec4-8* mutation (Goud *et al.*, 1988).

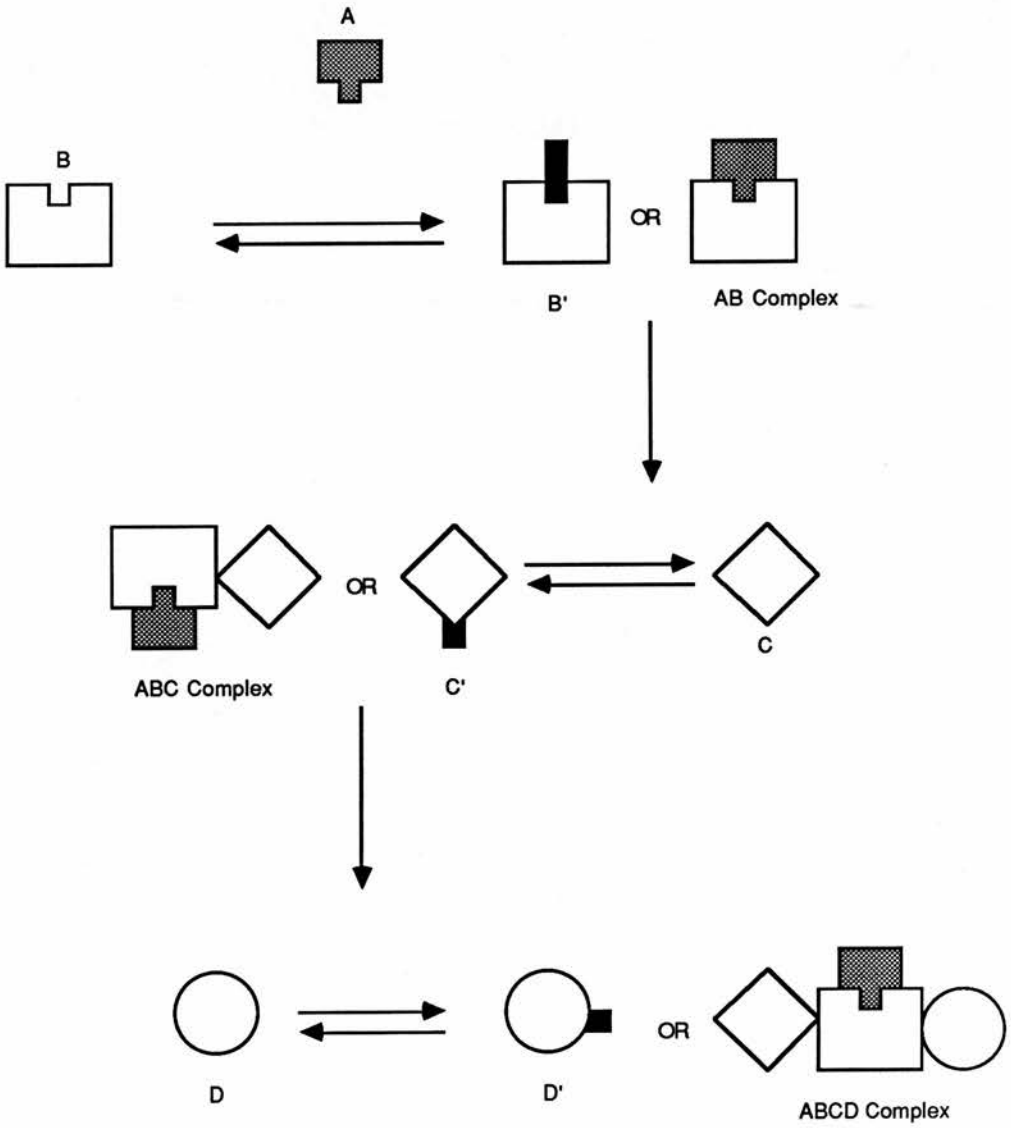

A
B
OR
B'
AB Complex
OR
C
ABC Complex
C'
D
D'
OR
ABCD Complex

Figure 9.2 **Control of mitosis in *Schizosaccharomyces pombe*.**

This model has been proposed by Russell and Nurse (1987). Three elements of this control circuit are proposed to be protein kinases (P.K.); for the *wee1*$^+$ and *nim1*$^+$ gene products, this is based upon sequence similarities with other protein kinases, but biochemical evidence has been obtained in support of the *cdc2*$^+$ gene product being a protein kinase (Simanis and Nurse 1986). See text for further discussion.

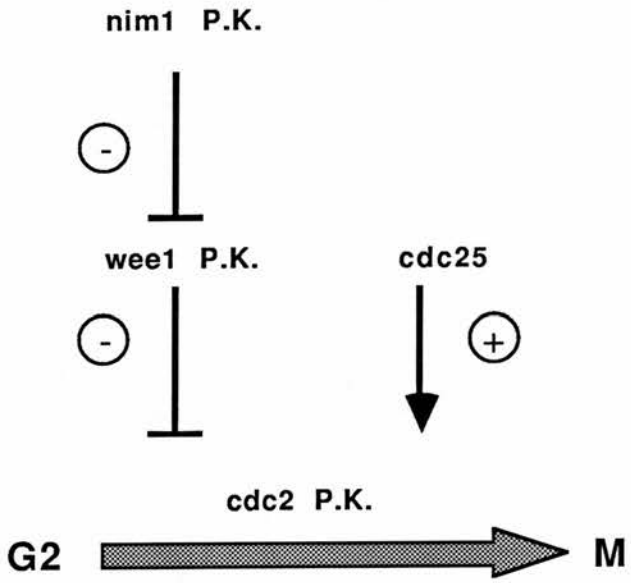

nim1 P.K.
-
wee1 P.K.
-
cdc25
+
cdc2 P.K.
G2
M

Figure 9.3 **Functional relationship between Sec1p and Nim1p.**

Model A suggests that Nim1p and Sec1p sequentially interact with protein X, and that interaction of Sec1p with protein X is dependent upon the phosphorylation state of protein X. In this view, Nim1p binds to protein X and phosphorylates it; phosphorylation results in a conformational change of protein X, allowing interaction with Sec1p.

Model B indicates that Sec1p can interact with protein X without prior phosphorylation of the substrate. In this view competition for protein X would exist between Sec1p and Nim1p. Formation of a Sec1p-protein X complex would block phosphorylation of protein X by Nim1p.

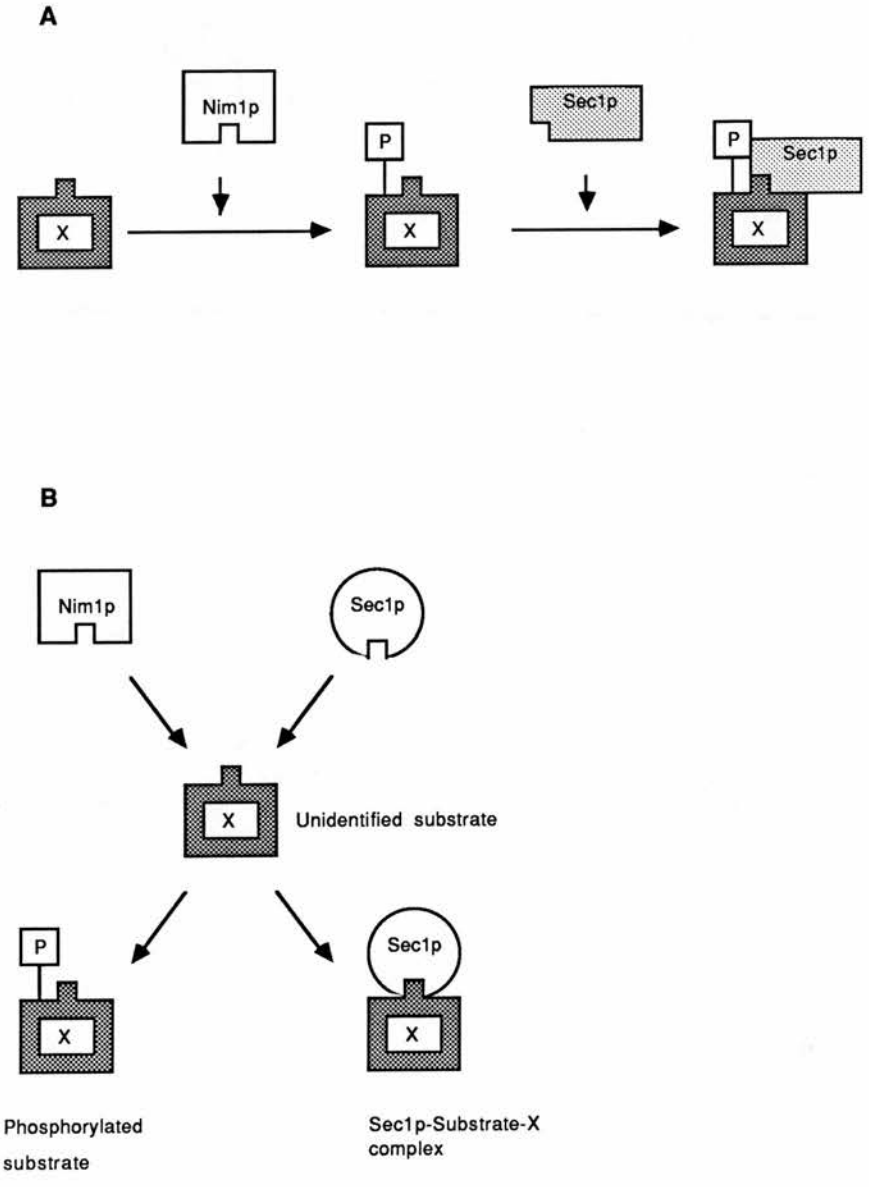
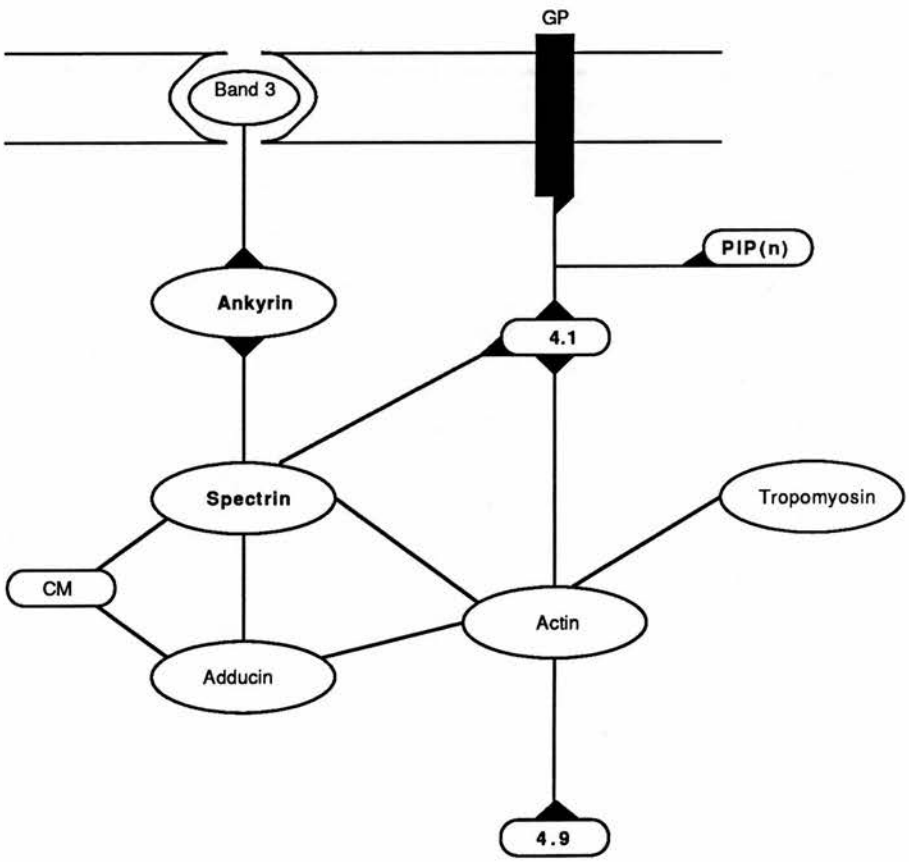

A
Nim1p
P
X
Sec1p
P
Sec1p
X
B
Nim1p
Sec1p
X
Unidentified substrate
P
X
Sec1p
X
Phosphorylated
substrate
Sec1p-Substrate-X
complex

Figure 9.4 **The erythrocyte cytoskelton**.

This diagram is a schematic illustration of the erythrocyte cytoskeleton. Proteins which are known to be phosphorylated are in bold text. Those proteins that interact with each other are connected by lines, and arrowheads indicate those interactions that are influenced by phosphorylation. PIP_n, phosphatidylinositol phosphates; CM, calmodulin; GP, glycophorin.


GP
Band 3
PIP(n)
Ankyrin
4.1
Spectrin
Tropomyosin
CM
Actin
Adducin
4.9

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